NEW INSIGHTS INTO BIOMOLECULE POLYMERIZATION UNDER PLAUSIBLE PRIMORDIAL EARTH CONDITIONS: IMPLICATIONS FOR THE ORIGIN OF LIFE

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By

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NEW INSIGHTS INTO BIOMOLECULE POLYMERIZATION UNDER PLAUSIBLE PRIMORDIAL EARTH CONDITIONS: IMPLICATIONS FOR THE ORIGIN OF LIFE

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To my parents, Tom and Carol, and my sisters, Amy and Kristen,
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LIST OF ABBREVIATIONS

amu	Atomic Mass Unit
Da	Dalton
mDa	Millidalton
m/*z*	Mass-to-charge ratio
g	Gram(s)
mg	Milligram(s)
wt%	Weight Percent
mol	Mole
mmole	Millimole
ppb	Parts-per-billion
M	Molar Concentration
mM	Millimolar Concentration
atm	Atmospheres
mb	Millibar(s)
mmHg	Millimeters of Mercury
Km	Kilometer(s)
cm	Centimeter(s)
µm	Micrometer(s)
nm	Nanometer(s)
L	Liter(s)
mL	Milliliter(s)

µL	Microliter(s)
v:v	Volume-to-volume ratio
s	Second(s)
ms	Millisecond(s)
min	Minute(s)
hr	Hour(s)
bya	Billion Years Ago
Ga	Billions of Years Before Present
°C	Degrees Celsius
K	Degrees Kelvin
V	Volt(s)
eV	Electron Volt(s)
kV	Kilovolt(s)
MΩ cm	Mega Ohm-Centimeter(s)
N	Normality (Equivalent Concentration)
S	Sensitivity
dt	Drift Time
AC	Alternating Current
HILIC	Hydrophilic Interaction Chromatography
HPLC	High Performance Liquid Chromatography
UPLC	Ultra Performance Liquid Chromatography
IEC	Ion Exchange Chromatography
IPC	Ion-Pair Chromatography

k'	Column Capacity Ratio
OPA/NAC	*ortho*-pthaldialdehyde/N-acetyl-L-cysteine
RT	Retention Time
EIC	Extracted Ion Chromatogram
FD	Fluorescence Detector
ESI	Electrospray Ionization
IMS	Ion Mobility Spectrometer
CID	Collision-Induced Dissociation
MRM	Multiple Reaction Monitoring
QqQ-MS	Triple Quadrupole Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
TOF-MS	Time-of-Flight Mass Spectrometry
Q-TOF	Quadrupole Time-of-Flight
UPLC-Q-TWIMS-TOF-MS	Ultra Performance Liquid Chromatography-Traveling Wave Ion Mobility Spectrometry-Time-of-Flight Mass Spectrometry
GC-MS	Gas Chromatography-Mass Spectrometry
FT-ICR-MS	Fourier Transform Ion Cyclotron Resonance Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantitation
CC	Carbonaceous Chondrite
ED	Electric Discharge

MU	Miller-Urey
STD	Standard
UV	Ultraviolet
Fe	Iron
Ni	Nickel
P	Phosphorus
Ar	Argon
CH_4	Methane
CO	Carbon Monoxide
CO_2	Carbon Dioxide
COS	Carbonyl Sulfide
H_2O	Water
H_2S	Hydrogen Sulfide
N_2	Nitrogen
NH_3	Ammonia
O_2	Oxygen
SO_2	Sulfur Dioxide
HCl	Hydrochloric Acid
ddHCl	Double Distilled Hydrochloric Acid
HCN	Hydrogen Cyanide
KOH	Potassium Hydroxide
NH_4^+	Ammonium Ion
$NaHCO_3$	Sodium Bicarbonate

NaOH	Sodium Hydroxide
NH_4Fo	Ammonium Formate
NH_4OAc	Ammonium Acetate
NH_4OH	Ammonium Hydroxide
CYA	Cyanamide
DICYA	Dicyandiamide
MA	Methylamine
AA	Amino Acid
Ala, A	Alanine
Arg	Arginine
Asn	Asparagine
Asp, D	Aspartic Acid
Cys	Cysteine
Gln	Glutamine
Glu, E	Glutamic Acid
Gly, G	Glycine
His	Histidine
Ile	Isoleucine
Ise	Isoserine
Iva	Isovaline
Leu	Leucine
Lys	Lysing
Met	Methionine

Nle	Norleucine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
α-ABA	alpha-Aminobutyric Acid
β-ABA	beta-Aminobutyric Acid
γ-ABA	gamma-Aminobutyric Acid
α-AIB	alpha-Aminoisobutyric Acid
β-AIB	beta-Aminoisobutyric Acid
β-Ala	beta-Alanine
EACA	epsilon-Aminocaproic Acid
AHA	alpha-Hydroxy Acid
OA	Organic Acid
VA	Volatile Acid
DKP	Diketopiperazine
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
TOC	Total Organic Carbon
PTFE	Polytetrafluoroethylene

SUMMARY

The search for the chemical origin of life is one of the greatest, and most exciting, challenges of our time. The formation of molecules considered important for life has been studied for over 200 years. However, the exploration of environmental conditions that could have facilitated the prebiotic chemical evolution necessary to generate such compounds, only began in the 20th century. It was not until the 1950s that these two areas of research were first merged, when the iconic Miller-Urey experiment demonstrated the synthesis of amino acids upon simulating a primitive ocean-atmosphere system using very simple starting materials. Since Miller's pioneering work, tremendous advancements in analytical chemistry have been made, greatly enhancing the chemical detection capabilities that origin of life chemists have access to. In recent years, a number of original, archived, 1950s Miller samples have been discovered, generating renewed interest in his seminal work. These old samples have since been analyzed, some for the first time, with state-of-the-art instrumentation. The findings uncovered a cornucopia of monomer biomolecules formed under such possible early Earth environments as volcanic vent systems, yet many went undetected in the 1950s due to analytical limitations.

While much of the work in the field of prebiotic chemistry has focused on the synthesis of monomer organics, the polymerization of these simple biomolecules has emerged as a leading challenge facing the origin of life community. Consequently, the studies herein investigate the transition from organic building blocks to simple peptides.

A standard protocol for the execution of prebiotic simulation experiments was first established for the testing of a variety of plausible primitive conditions. The results

of this work hold important implications for chemical evolution in environmental scenarios that could have been found on the early Earth and other planetary bodies. To illustrate, Miller-Urey experiments performed here produced amino acids similarly found in Titan simulation reactions, suggesting that such electric discharge reactions are good analogs of hydrated Titan tholins, and that biomolecule synthesis is robust under drastically different simulated environmental conditions.

Concomitantly, new analytical techniques were developed using a suite of approaches, including liquid chromatography, ion mobility spectrometry, high resolution mass spectrometry, and tandem mass spectrometry. The complex mixtures produced by the experiments performed here were then analyzed for polymerization products using the developed methods.

In addition to executing original experiments, archived extracts from Miller's previously unreported 1958 cyanamide spark discharge experiment were analyzed for amino acids and simple peptides to evaluate the possibility of inducing amino acid polymerization and peptide synthesis via condensing reagents under potential prebiotic conditions. Miller's original cyanamide spark discharge experiment showed that cyanamide readily induces amino acid polymerization, and repeating the experiment confirmed the synthesis of peptides. Aqueous solution heating experiments revealed that the observed polymerization chemistry was spurred on by the presence of the intermediates of amino acid synthesis and the dimerization of cyanamide under basic conditions.

To assess the plausibility of the co-polymerization of amino acids and their α-hydroxy acid analogs to form depsipeptides, thereby avoiding the diketopiperazine trap,

an ultra performance liquid chromatography-tandem mass spectrometry method was developed to identify and quantify α-hydroxy acids in a single run. When applied to mixtures generated here, this method helped discover that the monomers glycolic acid, glycine, lactic acid, alanine, malic acid, aspartic acid, α-hydroxyglutaric acid, and glutamic acid are the most pertinent for future abiotic depsipeptide synthesis studies.

Finally, prebiotic experimental samples were analyzed for signs of the *in-situ* synthesis of depsipeptides via customized time-nested analytical techniques. This work marked the first development of an analytical platform to analyze simple depsipeptides generated from prebiotic simulation experiments and subsequent mimicked environmental cycling. This new analytical technique used ion-pair chromatography, ion mobility spectrometry, and high resolution tandem mass spectrometry to identify target depsipeptides. This exploration revealed evidence for the tentative identification of the glycolic acid-aspartic acid/malic acid-glycine didepsipeptides, and the malic acid-aspartic acid didepsipeptide. These findings suggest that peptides can be formed, without succumbing to the diketopiperazine trap, simply by exposing complex mixtures generated from prebiotic simulation experiments to environmental cycling.

The culmination of this thesis expands our knowledge of the range of chemical and environmental conditions that could have supported the synthesis of simple and more complex biomolecules on the primordial Earth and elsewhere.

CHAPTER 1: INTRODUCTION

1.1 Abstract

The origin of life is one of the most mysterious challenges in science, and is an area of intense research. Long before Charles Darwin's hypothesis that a "warm little pond" may have served as a site for the origin of life, researchers studied the abiotic synthesis of organic molecules. Later, scientists pontificated about environments that could have existed on the early Earth, and the suitability of these environments to foster the origin of life. Until the pioneering 1950s work of Stanley Miller, however, the origin of life was considered merely a theoretical curiosity. Miller's transformative research brought legitimacy to the study of the origin of life, and ushered in a new field of research called prebiotic chemistry, the study of the chemical molecules and reactions that could have set the stage for the origin of life. The analytical techniques Miller used were crude by today's standards, but analytical chemistry has drastically improved since, providing significantly better detection capabilities. Modern analytical techniques were applied to recently discovered, archived, 1950s Miller samples, some of which went unreported. Re-analysis of Miller's classic, volcanic, and silent experimental samples revealed a much wider array of biomolecules than Miller detected. When applied to samples from Miller's previously unreported 1958 hydrogen sulfide experiment, new analytical methods highlighted that biochemically important sulfur amino acids, like methionine, could have been synthesized under primitive Earth conditions. While much research has been completed to date, this chapter will overview a select collection of

early explorations into origin of life-, and prebiotic-, chemistry that have paved the way for decades of future research efforts to aid the search for the origin of life.

1.2 The Importance of Studying the Origin of Life

Arguably the single-most intrinsically fascinating scientific question that remains unanswered pertains to the nature of the origin of life. Deciphering a plausible route by which life could have originated on Earth would hold vast implications for the field of prebiotic chemistry and numerous other fields of study. The magnitude of the importance of this type of research finding would mandate its inclusion in introductory science textbooks, necessitating that these fundamental resources be categorically amended.

Studying the origin of life is an inherently multidisciplinary research endeavor, and therefore makes valuable contributions to a variety of fields. Organic chemistry is employed to understand chemical reactions that were likely important for generating primitive biomolecules. Such knowledge gained can provide new insight into previously unexplored mechanisms of organic synthesis.[1] Inorganic chemistry and physical chemistry are pivotal to understand the chemistry of minerals, which are thought to have expanded the prebiotic chemical inventory.[2] Analytical chemistry is a valuable tool for tackling questions germane to the origin of life, and numerous other challenges. For example, developing new techniques can aid small molecule analysis in prebiotic reactions, but are also of value for detecting narcotics, explosives, environmental pollutants, and chemical warfare agents.[3] Lastly, biochemistry is implemented in origin of life studies, including those regarding ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) structure stability in possible prebiotic solvents, for example.[4] Results of

such studies are useful for self-assembly, medicinal chemistry, and nanotechnology, to name a few.[5, 6]

1.3 Early Abiotic Synthesis of Molecules Important for Life

Prebiotic chemistry is the study of the molecules and chemical reactions that likely contributed to the formation of complex biopolymers on the early Earth. For such structures to give way to life, they must have had the ability to undergo polymerization, replication, and self-organization the under abiotic conditions that likely occupied the primitive Earth. The chemical conditions on the primordial Earth at the time of the origin of life are not explicitly known, providing innate challenges in establishing rigorous constraints on prebiotic plausibility with respect to organic synthesis. However, within the origin of life community, there exists an understanding that for a chemical synthesis to be considered prebiotically plausible, the following 3 criteria should be assessed:

1) Starting materials should have reasonably been present on the early Earth.
2) Chemical reactions should be able to take place, either in water (H_2O), or in the gaseous or solid phases in the presence of simple molecules that could have reasonably been present on the primordial Earth.
3) Reaction products forming from the aforementioned starting materials should be generated at quantities considered to be sufficiently large and robust to facilitate meaningful chemistry within an abiotic context.

With these constraints in mind, much can be gleaned by examining important milestones in the history of abiotic organic synthesis, a realm of research that has taken place in excess of 200 years. Some of the most noteworthy examples of such achievements in organic synthesis include the abiotic formation of nucleobases,[7, 8] urea,[9]

amino acids (AAs),[10] and sugars[11, 12] using simple starting materials that could have reasonably been present on the early Earth.

1.3.1 *The Abiotic Synthesis of Nucleobases*

Nucleobases are nitrogenous organic compounds broken up into two categories: pyrimidines and purines. Pyrimidines are six-membered, aromatic heterocyclic organic molecules containing two nitrogen atoms at the 1 and 3 positions (Figure 1.1.A). Purines are also aromatic heterocyclic organic compounds, but are composed of a pyrimidine linked to an imidazole structure (Figure 1.1.B). When linked to a 5-carbon sugar, nucleobases make up the building blocks of RNA and DNA, called nucleosides.

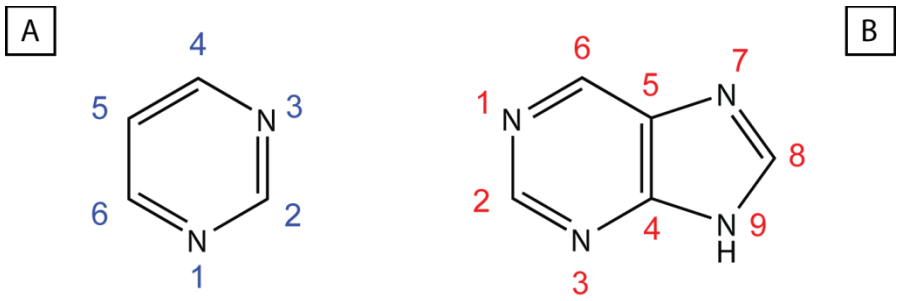


Figure 1.1 General structures of nucleobases of the pyrimidine (A) and purine (B) variety.

French chemist Joseph Louis Proust is thought to have executed the first laboratory synthesis of nucleobases in the early 1800s from aqueous solutions of hydrogen cyanide (HCN) under basic conditions.[7, 8] In 1960, researchers began to think one of Proust's products could have been the purine, adenine. Catalonian biochemist Joan Oró reported adenine generation upon heating aqueous solutions of ammonium cyanide below 100 °C.[13] Later, Oró and Kimball[14] demonstrated the base-catalyzed condensation of HCN in the presence of aqueous ammonia to produce the AAs glycine, alanine, and

aspartic acid. HCN has since been formed in prebiotic reactions,[15] and found in the interstellar medium.[16] These collective findings contribute to the consideration of HCN as a prebiotically plausible precursor to nucleobases.

1.3.2 *The Abiotic Synthesis of Urea*

Urea is another very simple monomer molecule that has been shown to be an active reagent in the generation of various biomolecules. Nearly two centuries ago, urea synthesis was achieved by German chemist Friedrich Wöhler.[9] Wöhler found that urea can immediately be formed simply by boiling ammonium cyanate, a species that is produced from the reaction of ammonium and cyanate.[17] Ammonium is formed from the dissolution of ammonia gas (NH_3) in H_2O, and cyanate can be generated via the electric discharge (ED) of hydrogen (H_2), nitrogen (N_2), and carbon monoxide (CO) over an aqueous solution of sodium bicarbonate ($NaHCO_3$).[18] Later, Liebig and Wöhler[19] observed that urea forms less spontaneously when gently evaporating aqueous cyanate.

One important reaction urea participates in leads to the formation of cytosine, a pyrimidine nucleobase (Figure 1.2). Urea reacts with cyanoacetaldehyde, a product of the hydrolysis of cyanoacetylene,[20] which readily forms from the ED of methane (CH_4) and N_2,[21] to form ~5% yields of cytosine.[20, 22, 23] Synthesis of cytosine from urea can also coincide with the formation of adenine from HCN,[24] and the hydrolysis of cytosine, to form uracil, another pyrimidine nucleobase.[25]

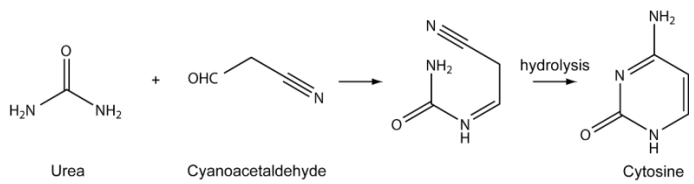


Figure 1.2 Synthesis of cytosine from the reaction of urea with cyanoacetaldehyde.

1.3.3 *The Abiotic Synthesis of AAs*

AAs are amine-containing carboxylic acids that have been dubbed the building blocks of life, as they are the monomer building blocks of proteins. Proteins are macromolecular biopolymers that perform a wide array of biological functions, from DNA replication,[26] to participating in metabolic reactions.[27] The abiotic synthesis of AAs was first reported in 1850 by German chemist Adolph Strecker.[10] Strecker showed that aldehydes, in the presence of NH_3 and HCN, can produce α-aminonitriles, AA precursors. And acid-catalyzed hydrolysis of the nitrile yields α-AAs (Figure 1.3). In this reaction, aldehydes could have easily been formed on the early Earth, given their rapid generation from ED experiments sparking a gas mixture of H_2O, H_2, NH_3, and CH_4.[28]

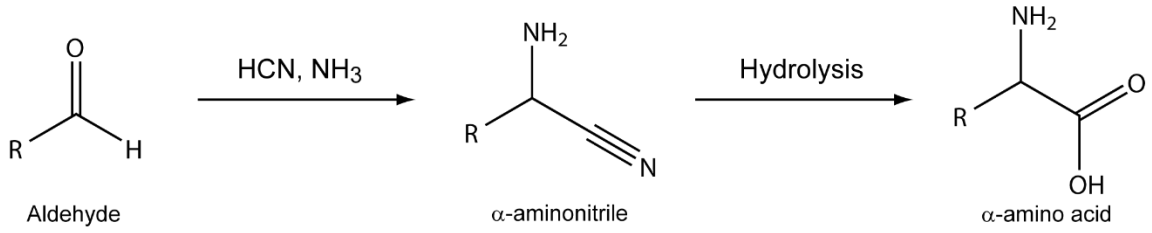


Figure 1.3 The Strecker synthesis of AAs.

1.3.4 *The Abiotic Synthesis of Sugars*

Sugars hold significant importance for the study of the origin of life, and are critical components of the structures of RNA and DNA. When linked up to a phosphate group, sugars help make up the sugar-phosphate backbone of nucleic acids. The abiotic synthesis of sugars was first revealed by Russian chemist Alexander Butlerow in 1861.[11, 12] Butlerow's seminal work, known as the formose reaction, is the base-catalyzed reaction of formaldehyde to yield sugars, including ribose. In addition to a base, the

reaction is carried out in the presence of a divalent cation, such as calcium, as chelating ions possess important stabilizing effects. It has since been observed that the particular metal ion used in the reaction can be very influential. To illustrate, sodium hydroxide (NaOH) or potassium hydroxide are relatively ineffective in facilitating the formose reaction, while calcium hydroxide is much more efficient.[29] It has also been reported that under some circumstances, when using the appropriate metal ion, the formose reaction can even be catalyzed under acidic regimes.[30]

1.4 Possible Primordial Earth Environmental Scenarios

The environmental conditions on Earth at the time of the origin of life are inherently challenging to constrain. Consequently, it is a topic that has long-since puzzled some of the most revolutionary scientists, like Charles Darwin. Darwin famously hypothesized of a "warm little pond" in an 1871 letter to his friend and botanist, Joseph Hooker, which could have existed on the early Earth and harbored life. Darwin thought such an environment might have possessed essential ingredients necessary for life, including energy and simple compounds that could have interacted with one another in a dynamic system to give rise to complex molecules, and eventually the origin of life.

However, it wasn't until the 1920s when Darwin's warm little pond idea was expanded upon. In 1924, Russian biologist Alexander Oparin published a tremendously influential book.[31] In his book, Oparin speculated that the early Earth's atmosphere contained reduced gases, which could have facilitated the formation of organic compounds in the primitive ocean. More specifically, Oparin thought that as the primordial Earth cooled, carbon that was previously in a bound form, could have become liberated to react with hydrogen and form hydrocarbon species. Oparin suggested that

organic compounds, such as AAs were not far behind, as their syntheses could have been facilitated by the presence of hydrogen and nitrogen, leading to the formation of necessary precursors, such as NH_3 and cyanide. Once these organic molecules were generated, Oparin noted that they could have undergone further chemistry in the early oceans, to produce coacervates, and eventually give rise to primitive life forms.

Near the same time as the publishing of Oparin's hypothesis, British geneticist John Haldane published his own, very similar ideas of how life evolved on Earth.[32] Haldane noted that upon the hardening of the molten Earth's surface, molecular oxygen (O_2) was unlikely to be present in the atmosphere, as O_2 is largely a product of photosynthesis. Instead, Haldane suggested the atmosphere was largely composed of NH_3 and carbon dioxide (CO_2). Without O_2, a protective stratospheric ozone layer would not have formed to shield the Earth's surface from ultraviolet (UV) radiation. Haldane emphasized such direct energy would have acted upon a primitive atmosphere of H_2O, CO_2, and NH_3 to serve as a powerful source of energy to drive the chemical synthesis of biologically relevant molecules, such as sugars and protein building blocks.

The Oparin-Haldane hypothesis, as it was called, was tremendously foresightful. These men reached their conclusions, not based on direct evidence, but based on their conceptions of early Earth conditions and how the components of the primitive Earth could have conceivably contributed to the origin of life. The Oparin-Haldane hypothesis became known as the Primordial Soup Theory, which collectively stated that the primitive oceans contained organic compounds that may have undergone polymerization, self-replication, and evolution to ultimately yield the formation of primitive organisms.

The Primordial Soup Theory was further elaborated upon by American Chemist Harold Urey in 1952,[33] when he expressed his thoughts on the physical and chemical conditions that may have existed on the early Earth at the time of the origin of life. Urey reasoned that H_2 was likely an abundant gas in the primitive atmosphere. In such a case, H_2 would be available to reduce carbon-bearing species to CH_4, oxygen-bearing species to H_2O, and sulfur-bearing species to hydrogen sulfide (H_2S). Furthermore, depending on the temperature of the atmosphere, nitrogen-bearing species would have been present as NH_3 or N_2. Under high temperature conditions, H_2 would have readily escaped the Earth's atmosphere, rendering nitrogen-bearing species to be present largely as N_2. However, relatively low atmospheric temperatures would have caused H_2 to stay closer to the Earth's surface longer, allowing for the conversion of nitrogen-bearing species to NH_3 or ammonium salts. Due to Urey's thoughts on the presence of H_2 in the primordial atmosphere, he suggested that the gaseous species present at the time of the origin of life were likeliest to be H_2, H_2O, CH_4, and NH_3, or N_2.

Urey then turned his thoughts to relevant energy sources that could have facilitated chemical evolution, and sustained active metabolic processes of primitive living organisms. Urey noted that a major source of free energy would have been in the form of the absorption of incoming solar energy by CH_4 and H_2O in the high altitude regions of the atmosphere, resulting in photochemical processes. Such absorption of energy would have served the added benefit of protecting primitive organisms from harmful solar radiation, given the absence of a stratospheric ozone layer to otherwise protect the Earth's surface from these UV rays. Urey also emphasized that EDs could have been an important source of energy to generate numerous compounds on the early

Earth, based on the work of Glockle and Lind[34] on the electrochemistry of gases, and also partially because electrical storms could have been a common occurrence in a primitive reducing atmosphere. Consequently, Urey proposed that studies of the synthesis of organics produced by the action of UV light, or EDs, on CH_4 and H_2O, with the possible inclusion of H_2, warranted further consideration. It is worth noting that at approximately the same time as Urey proposed these experimental endeavors, similar, views were expressed independently by John Bernal[35].

1.5 The Miller-Urey (MU) Experiments

The combined views of Urey and Bernal significantly influenced subsequent work that remains perhaps the most groundbreaking study related to the origin of life. This work became known as the MU experiments, and were the first laboratory-based evaluation of the Primordial Soup Theory. In the early 1950s, Stanley Miller, then a graduate student at the University of Chicago, worked under the direction of Professor Harold Urey to examine the possible production of organic compounds under simulated early Earth conditions. Miller performed a series of experiments to mimic plausible primitive environments. One such experiment was the action of an ED on a primitive ocean-atmosphere system. This experiment became known as the Classic MU Experiment. A later study, known as the Miller Volcanic Spark Discharge Experiment, was performed in which a volcanic eruption, in the presence of lightning discharges, was imitated. Lastly, Miller studied the action of a non-visible, non-audible ED on possible gases of the early Earth, an experiment referred to as the Silent Electric Discharge Experiment. Exploring a variety of environmental conditions allowed for the evaluation of the differences in organic production between various reaction scenarios.

1.5.1 *The Classic MU Experiment*

In 1953, Stanley Miller published the synthesis of molecules important for life by simulating a primitive ocean-atmosphere system.[36, 37] Miller devised a dual-flask glass apparatus for this experiment (Figure 1.4). One flask had a capacity of 500 mL, was positioned at the bottom of the apparatus, and was filled with water to simulate the early Earth's ocean (a.k.a "the ocean flask"). The other flask had a capacity of 5 L, was positioned at the top of the apparatus, and was filled with the gases NH_3, CH_4, and H_2, which, at the time, were thought to represent the primitive atmosphere (a.k.a. "the atmosphere flask"). The atmosphere flask contained two tungsten electrodes, across which an ED was enacted using a Tesla coil, simulating a lightning discharge.

The two flasks were connected to each other via two glass tubes. One glass tube stemmed from the ocean flask, upward to the atmosphere flask. This tube allowed for transportation of water vapor from the ocean flask to the atmosphere flask, to simulate evaporation and an early atmosphere saturated with water vapor. The second tube traveled downward from the atmosphere flask, back to the ocean flask. Built into this tube was a condenser, which occupied a short stretch of tubing beneath the atmosphere flask, through which cold water continuously flowed. The cold water lowered the temperature of this region of the apparatus, and as gases came in contact with it, they would exit the gas phase and enter the condensed phase. The resultant liquid then traveled back into the ocean flask, simulating precipitation, or river runoff. A U-tube design was also implemented into this tube, to control the flow of material back into the ocean flask. The experiment was started by gently boiling the water in the ocean flask and simultaneously sparking the gases in the atmosphere flask.

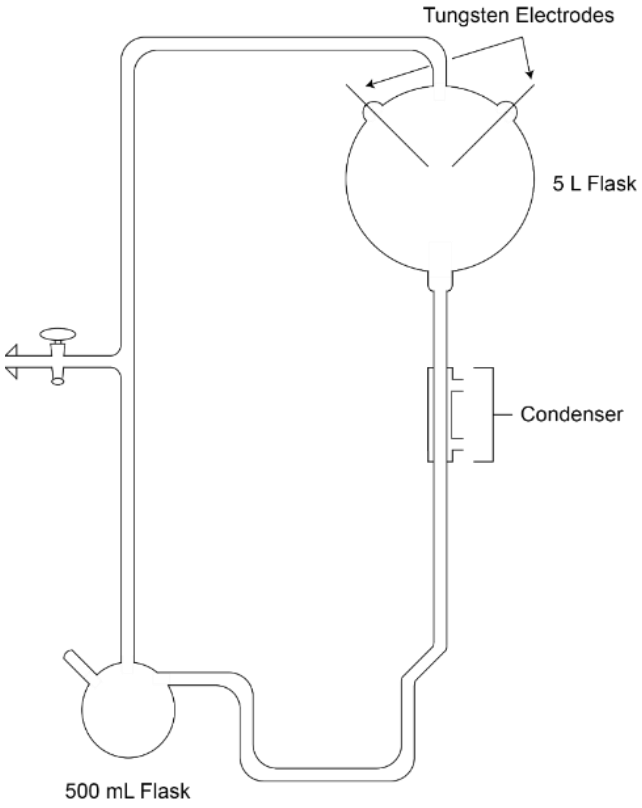


Figure 1.4 Schematic of the dual-flask, glass apparatus used in the classic MU experiment. Figure is adapted, with permission, from Parker, E. T., Cleaves, J. H., Burton, A. S., Glavin, D. P., Dworkin, J. P., Zhou, M., *et al.* Conducting Miller-Urey Experiments. *J. Vis. Exp.* (83), e51039, doi:10.3791/51039 (2014).

After 1-2 days of sparking, the water changed colors from clear, to yellowish-brown. The experiment ran for 7 days before the heating and sparking were stopped. At the completion of the experiment, the water in the ocean flask was dark brown, and the inner walls of the apparatus were covered by a dark, oily residue. Such a prominent, observed transformation indicated that chemistry was clearly happening; chemical bonds were being broken and formed throughout the experiment, producing compounds that were not originally present in the experimental starting conditions.

To answer the question of what was formed by the experiment, Miller turned to paper chromatography with ninhydrin detection, and the melting points of derivatives to

help confirm compound identification and quantitation. Miller's analyses revealed that the experiment, remarkably producing relatively high concentrations of a specific class of molecules, AAs. Analysis of an extract taken after 2 days of sparking showed a small spot for glycine on the paper chromatogram.[38] Analyzing an extract taken after 7 days of sparking showed a much more intense spot for glycine on the paper chromatogram, along with 6 additional spots, representative of other AAs. Miller identified 4 of the other 6 spots as alanine, β-alanine, aspartic acid, and α-aminobutyric acid. In addition to these primary AAs, Miller detected the secondary AA, sarcosine.

The classic MU experiment showed that an early Earth environment could be imitated in the laboratory using the simplest of starting materials, and in doing so, form AAs, the building blocks of life. Prior to Miller's pioneering work, the study of the origin of life was mere speculation. Miller's efforts brought legitimacy to the study of the origin of life, and ushered in the era of prebiotic chemistry.

1.5.2 *The Miller Volcanic Spark Discharge Experiment*

Miller's volcanic experiment was similar to the classic experiment, but received less notoriety. Miller set out to evaluate if island-arc systems could have been viable locations for the formation of organic molecules on the early Earth. To do this, an apparatus similar to that of the classic apparatus was constructed, except the atmosphere flask of the volcanic apparatus was positioned directly over the ocean flask. Additionally, an aspirator was positioned between the ocean flask and the atmosphere flask (Figure 1.5), to inject a jet of steam that interacts directly with the spark discharge. By doing so, the apparatus simulated a volcanic eruption in the presence of lightning-rich air, a phenomenon common on Earth today[39] and possibly throughout Earth's history.

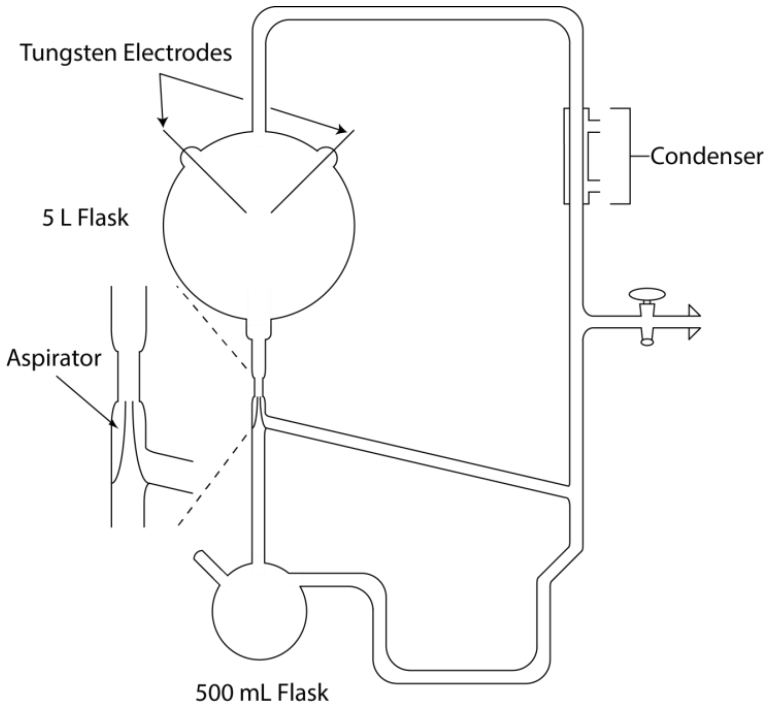


Figure 1.5 Schematic of the dual-flask glass apparatus used in Miller's volcanic spark discharge experiment.

Miller initiated the volcanic experiment by gently boiling the water in the ocean flask and simultaneously sparking a gas mixture of CH_4, NH_3, H_2O, and H_2.[37] This experiment carried on for 7 days before samples were collected and analyzed by paper chromatography with ninhydrin detection, and melting points of derivatives.

Miller first reported the results of his volcanic spark discharge experiment in 1955,[37] where he detailed the detection and quantification of 5 AAs: glycine, alanine, β-alanine, α-aminobutyric acid, and the secondary AA, sarcosine. Glycine and alanine were overwhelmingly the most abundant AAs formed, at 55 x 10^5 moles and 36 x 10^5 moles, respectively. Sarcosine was the least abundantly formed AA, at 2 x 10^5 moles.

The analyses uncovered the production of a similar set of AAs, and respective abundances, as produced in the classic experiment. The implication of this experiment is

that primordial volcanic systems could have synthesized biomolecules, which might have collected and accumulated in nearby tidal pools, and undergone further processing.

1.5.3 *The Silent Electric Discharge Experiment*

In a third simulation of a primordial environment, Miller veered away from a scenario in which a spark discharge was the primary energy source. An apparatus very similar to the volcanic apparatus was used, except instead of equipping the atmosphere flask with two tungsten electrodes for the generation of a spark discharge, an inaudible electric discharge was used as the energy source.[37] An aspirator was positioned between the two flasks to facilitate circulation throughout the apparatus. A 15,000 volt transformer was used to generate the silent discharge by applying 96 watts of power to copper electrodes that were placed in a 50% sulfuric acid solution (Figure 1.6).

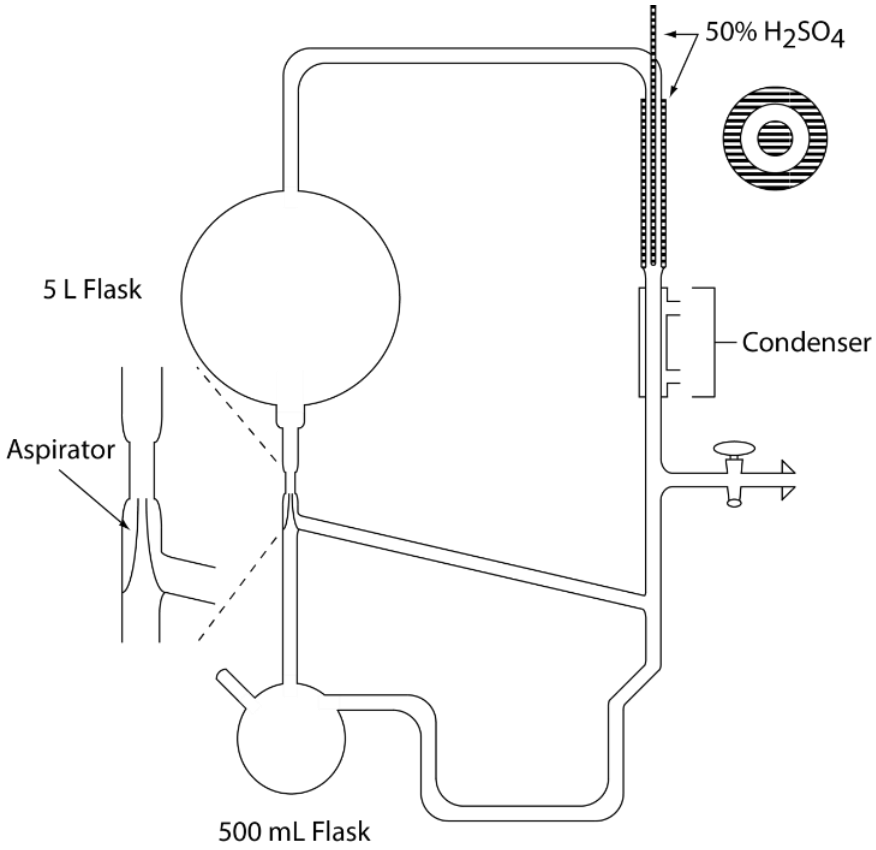


Figure 1.6 Schematic of the apparatus used in Miller's silent discharge experiment.

Miller implemented a silent discharge on a gas mixture of CH_4, NH_3, H_2O, and H_2 over a 7 day period. After the experiment concluded, liquid sample was collected and analyzed by paper chromatography with ninhydrin detection, and by the melting points of derivatives. The analytical results of the silent experiment were markedly different from those of the classic and volcanic experiments. The silent discharge experiment produced relatively large amounts of glycine and sarcosine, but relatively small abundances of other AAs, such as aspartic acid, glutamic acid, alanine, β-alanine, and α-aminobutyric acid. The results of this experiment, and the comparison of its products to those of the experiments that used a spark discharge, indicated that reaction conditions *do* matter. Clearly, the use of the spark discharge facilitated a more robust synthesis of biomolecules than did the use of a silent discharge.

1.6 The Evolution of Analytical Chemistry used to Study the Origin of Life

1.6.1 *Paper Chromatography with Ninhydrin Detection*

Miller prepared his samples for analysis by first purifying the discharge mixtures[37] using an ion exchange chromatography (IEC) cycle portrayed in Figure 1.7, which was modified from that described by Carsten.[40] Miller passed his samples through a Dowex-50 cation resin of the acid form (i.e. Column #1). In this step, all cations, including cations of strong bases, and ampholytes, including AAs, were retained, while anions and neutral species were discarded in the effluent. Ampholytes and weak bases, were recovered from the cation resin by elution using ammonium hydroxide (NH_4OH). The eluate was then passed through a Dowex-2 anion resin in its base form (i.e. Column #2), which discarded weak bases, but retained ampholytes, like AAs. AAs were recovered from Column #2 by elution with hydrochloric acid (HCl).

Miller then used an Amberlite IR 4B weakly basic ion exchange resin (acetate form, pH 5.0[41]) (i.e. Column #3) to retain acidic AAs eluting from Column #2, using the method described by Haas and Stadtman.[42] In the process, Column #3 discarded neutral and basic AAs. The acidic AAs were eluted from Column 3 using HCl. Upon the completion of this step, the acidic AAs were ready for analysis. Lastly, neutral and basic AAs were further purified using a Dowex-50 cation exchange chromatography column of the acidic form (i.e. Column #4). These AA species were retained by Column #4, while impurities were discarded. The AAs of interest were then eluted from Column #4 using HCl, at which point the neutral and basic AAs were ready for analysis.

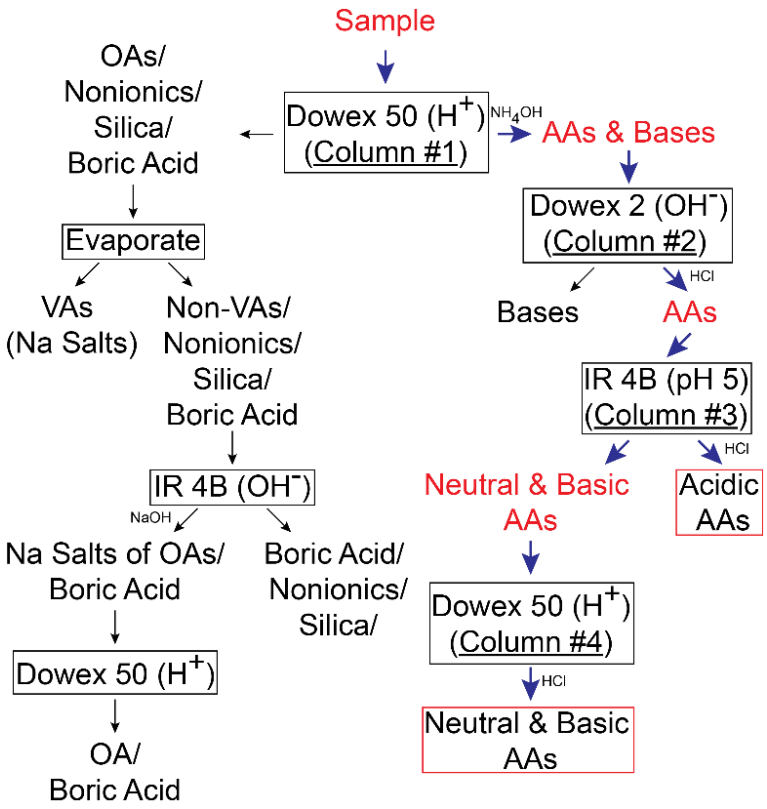


Figure 1.7 Ion exchange chromatography method used to prepare ED samples for analysis, as modified from[37]. Steps demarked by blue arrows are those Miller used to extract AAs. Solutions represented with red text are those that underwent the steps denoted by blue arrows. AA species denoted by a red box are those compounds successfully isolated from ED samples, prior to analysis. Here, OA = organic acid, and VA = volatile acid.

Paper chromatography was commonly used for early AA analyses of ED mixtures. A drop of the ED mixture was placed onto the bottom of absorbent paper. Typically, two buffers were used for elution of AAs, butanol-acetic acid-water, and phenol with 0.3%-0.5% NH_3.[36, 37] The aqueous-based-, and organic-based-, solvents were applied to adjacent sides of the paper chromatogram. The buffers traveled up the paper, carrying individual components of the sample mixture with them, depositing individual components of the mixture at characteristic locations along the paper chromatogram. After chromatographic separation, ninhydrin was then applied to the chromatogram to react with amines, and primary, or secondary, AAs. The reaction between ninhydrin and these amino-containing species produces the ninhydrin chromophore, Ruhemann's Purple (diketohydrindylidenediketohydrindamine), which absorbs ultraviolet light and reflects a deep blue/purple color, enabling the detection of AAs (Figure 1.8).

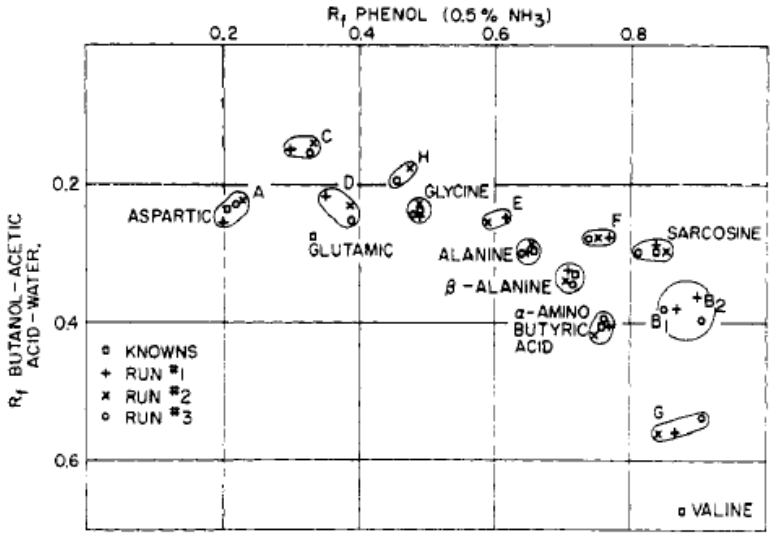


Figure 1.8 Paper chromatogram demonstrating the separation and detection of AAs in the MU Experiments. Here, Run #1 = classic MU experiment, Run #2 = volcanic spark discharge experiment, Run #3 = silent electric discharge experiment. Reprinted (adapted) with permission from (Miller, S. L. (1955) *Journal of the American Chemical Society*, 77(9), 2351-2361). Copyright (1955) American Chemical Society.

The manner in which ninhydrin reacts with AAs is shown in Figure 1.9, and has been discussed by Moore and Stein,[43] but will be briefly overviewed here. Ninhydrin (I), can rearrange to form 1,2,3-indantrione (II), which, in the presence of an AA (III), forms the common aldimine (IV). The aldimine then undergoes resonance stabilization and hydrolysis, to form an amine intermediate (VI). The intermediate amine can then react with another molecule of ninhydrin, to eventually form Ruhemann's purple (VII). This chromophore is formed by the abstraction of a highly acidic hydrogen (highlighted in green in Figure 1.9) located α to both two carbonyls and the central imine group. The removal of this hydrogen by hydroxide ion (also highlighted in green in Figure 1.9) produces a carbanion between two carbonyls, which can participate in charge distribution, giving Ruhemann's purple its chromophoric properties.

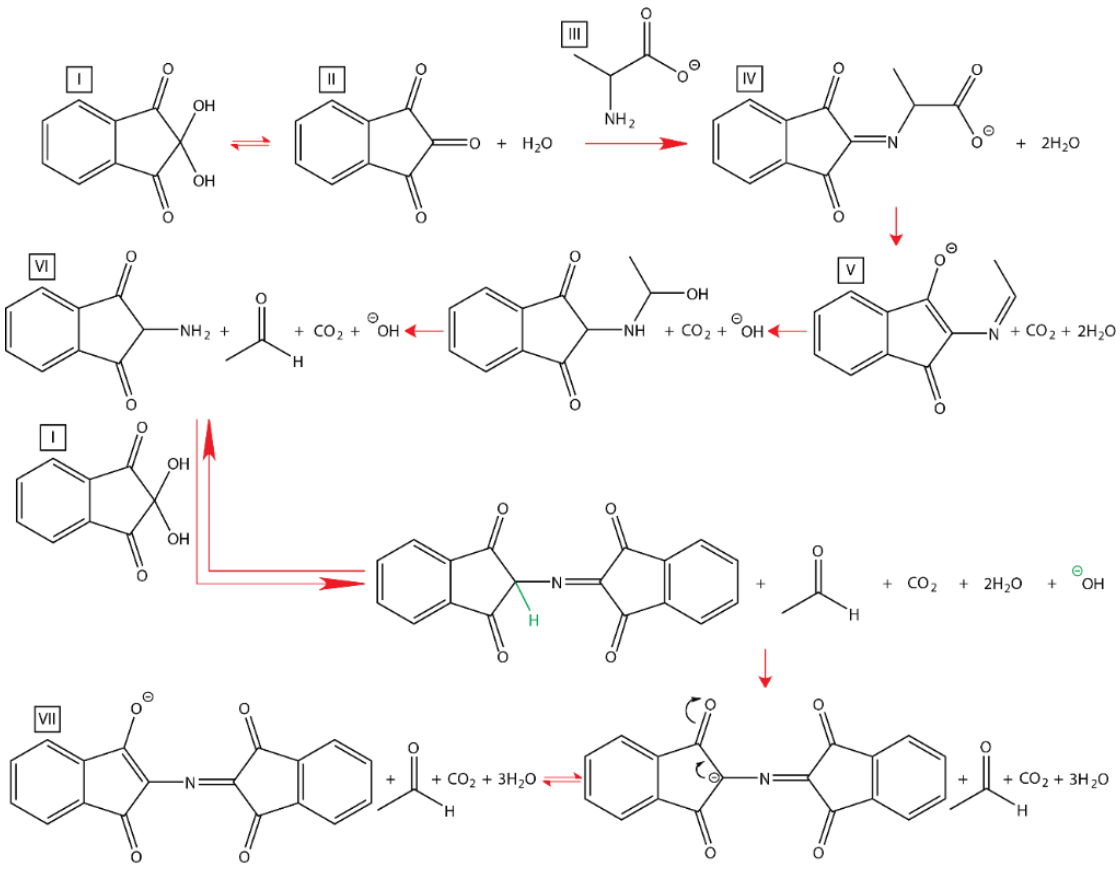


Figure 1.9 Mechanism of AA reaction with ninhydrin, adapted from Friedman and Williams[44] and Bottom et al.[45] In the mechanism presented here, I = ninhydrin, II = 1,2,3-indantrione, III = AA (alanine is shown as an example), IV = common aldimine, VI = amine intermediate, and VII = Ruhemann's Purple.

AA identities were confirmed by evaluation of mixed melting points. For example, preparing the *p*-toluenesulfonate of glycine[46] gives a reference melting point of 147.0 °C – 148.0 °C.[37] A glycine sample mixed with *p*-toluenesulfonate has a mixed melting point of 146.5 °C – 147.5 °C.[37] Additionally, alanine *p*-toluenesulfonate has a melting point of 140.0 °C – 140.5 °C, while an alanine sample with *p*-toluenesulfonate gives a mixed melting point of 140.0 °C – 141.0 °C.[37] The contrasting 133 °C mixed melting point of L-alanine *p*-toluenesulfonate[47] indicates alanine was racemic.

1.6.2 *High Performance Liquid Chromatography-Fluorescence Detection*

As the understanding of prebiotic chemistry advanced, so too, did analytical capabilities. In the 1950s, separation of complex mixtures was facilitated by relatively crude paper chromatography, or by very large chromatography columns (Figure 1.10). Drawbacks of large columns included: 1) the use of vast quantities of packing material and solvents, and 2) buffer flow rates were dictated by gravity.

Figure 1.10 Example of a chromatographic column commonly used in the 1950s. Note, the scientist in this image needs to stand on a step-ladder to reach the top of the chromatography column, so as to add eluent to the stationary phase of the column. Image courtesy of http://affinity-chromatography.tripod.com/ac.html.

In the 1960s, the first commercial high performance liquid chromatography (HPLC) unit was released.[48] HPLC quickly became a major workhorse for chemists, serving as an analytical staple between the 1970s and 1990s. HPLC contained specific improvements from more primitive chromatographic approaches. HPLC used small

particle (5 µm) packing material, pumps to control mobile phase flow rates, and much shorter column lengths to perform separations. Smaller sized packing material allowed for more precise interactions between target analytes in the mobile phase, and the packing material of the stationary phase. This enhanced analyte interaction improved peak shape, which, in turn, increased quantitative accuracy. Using pumps to dictate the flow rate of buffers through the column improved retention time consistency, which enabled more accurate identification of analytes based on retention time. Lastly, smaller columns allowed for separations to be performed on the order of tens of minutes, as opposed to hours, or days.

UV fluorescence detector (FD) systems were often coupled to HPLC units for analysis of prebiotic mixtures. HPLC-FD has been used to detect organics like polycyclic aromatic hydrocarbons[49, 50] and AAs.[51] To make biomolecules of prebiotic interest, such as AAs, fluoresce to enable detection by FD, derivatization agents, such as *ortho*-pthaldialdehyde/N-acetyl-L-cysteine (OPA/NAC) are often used. AAs were first derivatized with OPA/NAC by Aswad,[52] and later applied to geological[51, 53, 54] and meteoritic samples.[55] OPA/NAC is made by reacting *o*-pthaldialdehyde (also spelled *o*-pthalaldehyde) with N-acetyl-L-cysteine in a borate buffered solution (Figure 1.11).

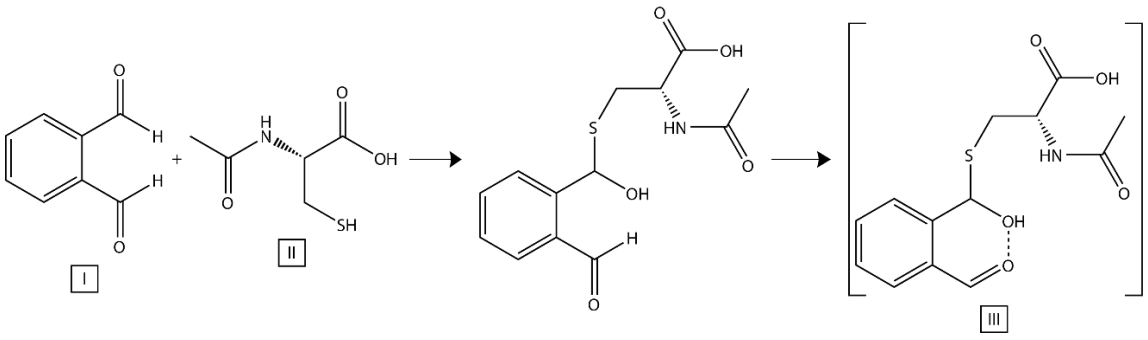


Figure 1.11 Mechanism of OPA (I) reacting with NAC (II) to form OPA/NAC (III).

Once OPA/NAC is formed, it can be used to detect amino-containing species like AAs and amines. OPA/NAC is capable of reacting with primary amines (Figure 1.12) to facilitate their detection and quantitation, and provides enantiomeric separation of AAs with chiral centers. Fluorescence detection of AAs via OPA/NAC derivatization is optimized when the FD is operated at an emission wavelength of 450 nm, and an excitation wavelength of 340 nm.[53]

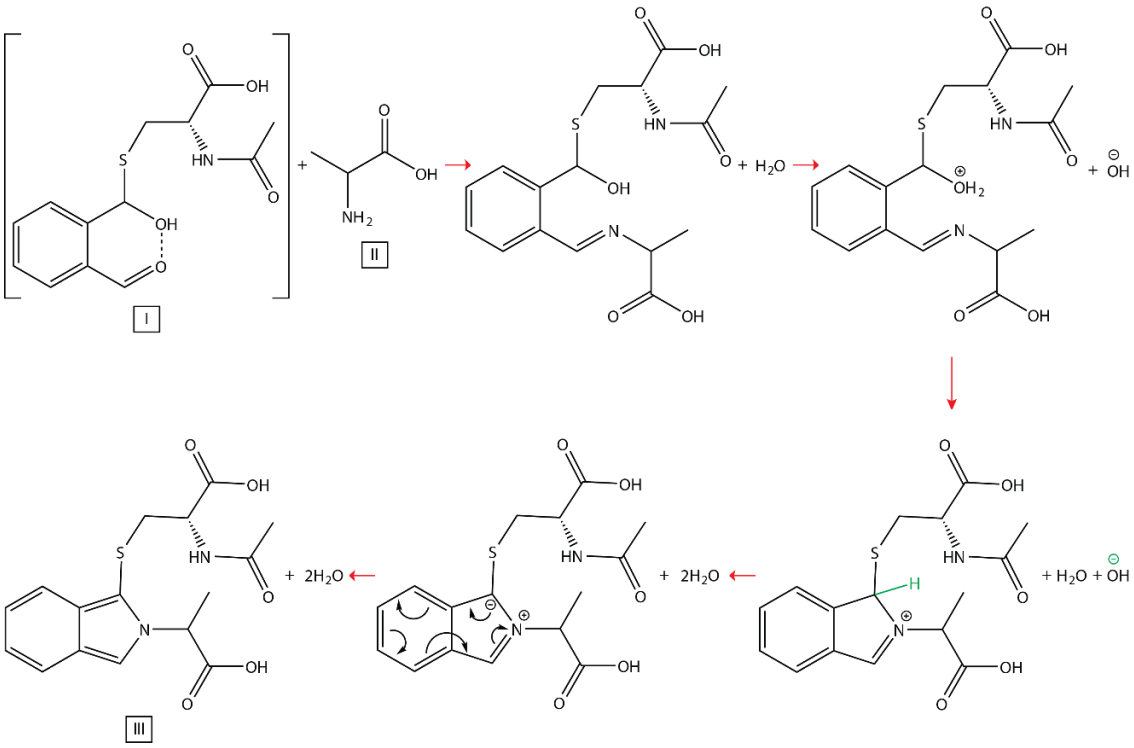


Figure 1.12 Reaction mechanism of OPA/NAC (I) reacting with an AA (II, alanine is shown as an example), to produce the detected fluorogen AA derivative (III). This mechanism is based on discussions provided in Simmons and Johnson.[56]

1.6.3 *Ultra Performance Liquid Chromatography-Mass Spectrometry*

In the early 2000s, a new type of liquid chromatography was unveiled, called ultra performance liquid chromatography (UPLC).[57] This new separation approach uses a 1.7

μm particle size stationary phase (~30% of that used for HPLC), and shorter columns than HPLC. The use of smaller particle sizes necessitated higher pressures to control eluent flow rates, and improved peak capacity and instrumental sensitivity. These improvements decreased analysis time to several minutes per run, in many cases.

Today, perhaps the most commonly used detector attached to UPLC systems based on mass spectrometry (MS). The first ever reported attempt to couple MS to an LC system was by Tal'roze et al.[58] Since then, researchers have developed a variety of ionization sources to aid in the analysis of mixtures by LC-MS, including a particle beam interface[59, 60] and atmospheric pressure ionization.[61] In the 1980s came the adaptation of electrospray ionization (ESI) to MS experiments by Fenn and coworkers.[62] ESI was rapidly incorporated into commercial LC-MS systems and remains a ubiquitous ion source used in LC-MS instrumentation today.

ESI-LC-MS has proven effective when analyzing a wide array of biomolecules relevant for the study of the origin of life. For example, ESI-UPLC-MS is routinely used when analyzing meteorites, which harbor primitive chemical records of our solar system, for AAs. Glavin and Dworkin[63] reported the use of UPLC coupled with time-of-flight mass spectrometry (TOF-MS) to analyze aqueously-altered meteorite samples for C_5 AAs. The findings of the study were that large L-enantiomeric excesses of isovaline existed in the CI meteorite Orgueil and the CM meteorite Murchison. This result suggests that the parent bodies of these meteorites may have been responsible for the enrichment of the L-enantiomer of α-dialkyl AAs, including isovaline, in the prebiotic chemical inventory, and potentially contributing to the prevalence of L-AAs in biology. UPLC-FD/TOF-MS was later employed by Burton et al.[64] to study AAs in thermally altered

meteorites that fell on the Antarctic ice sheet. The findings were that thermally altered carbonaceous chondrites (CCs), including type 3 CO and CV CCs, contain an AA inventory that largely consists of straight-chain, short AAs with amine terminal groups. This finding is in contrast to the analyses of aqueously altered meteorites that contain large concentrations of AAs, including complete structural diversity of these AAs, which is consistent with their formation by the Strecker synthesis mechanism. The short, amine terminal AAs found in thermally altered meteorites are not associated with Strecker synthesis, but are consistent with other cosmochemical mechanisms that may be at play.

In addition to AAs, other important biological monomers, such as nucleobases, have been targeted during origin of life studies using UPLC-MS. For example, Callahan et al.[65] reported the development of a UPLC-FD/TOF-MS method to detect nucleobases and nucleobase analogs in the Martian meteorite Roberts Massif 04262, in addition to AAs. A standard mixture of the nucleobases cytosine, guanine, and adenine could be detected at a concentration of 1 ppb, as well as the analogs purine, isocytosine, 2-aminopurine, 2,6-diaminopurine, 6,8-diaminopurine, and hypoxanthine. These species were not detected in Roberts Massif 04262, but the tentative detection of guanine (~0.1 ppb) was reported in Allan Hills 84001.

Combining UPLC and MS has proven to be valuable for the analysis of organic molecules within complex samples. Such methods enabled fast, sensitive, and robust analysis of various analytes in complex matrices to answer questions pertinent to prebiotic chemistry and the origin of life. Perhaps the most significant impact that UPLC-MS techniques have had on prebiotic chemistry pertain to their application to solutions generated from prebiotic experiments, including ED experiments performed by Miller.

1.7 Analysis of Previously Reported and Unreported Miller ED Experiments using Modern Analytical Techniques

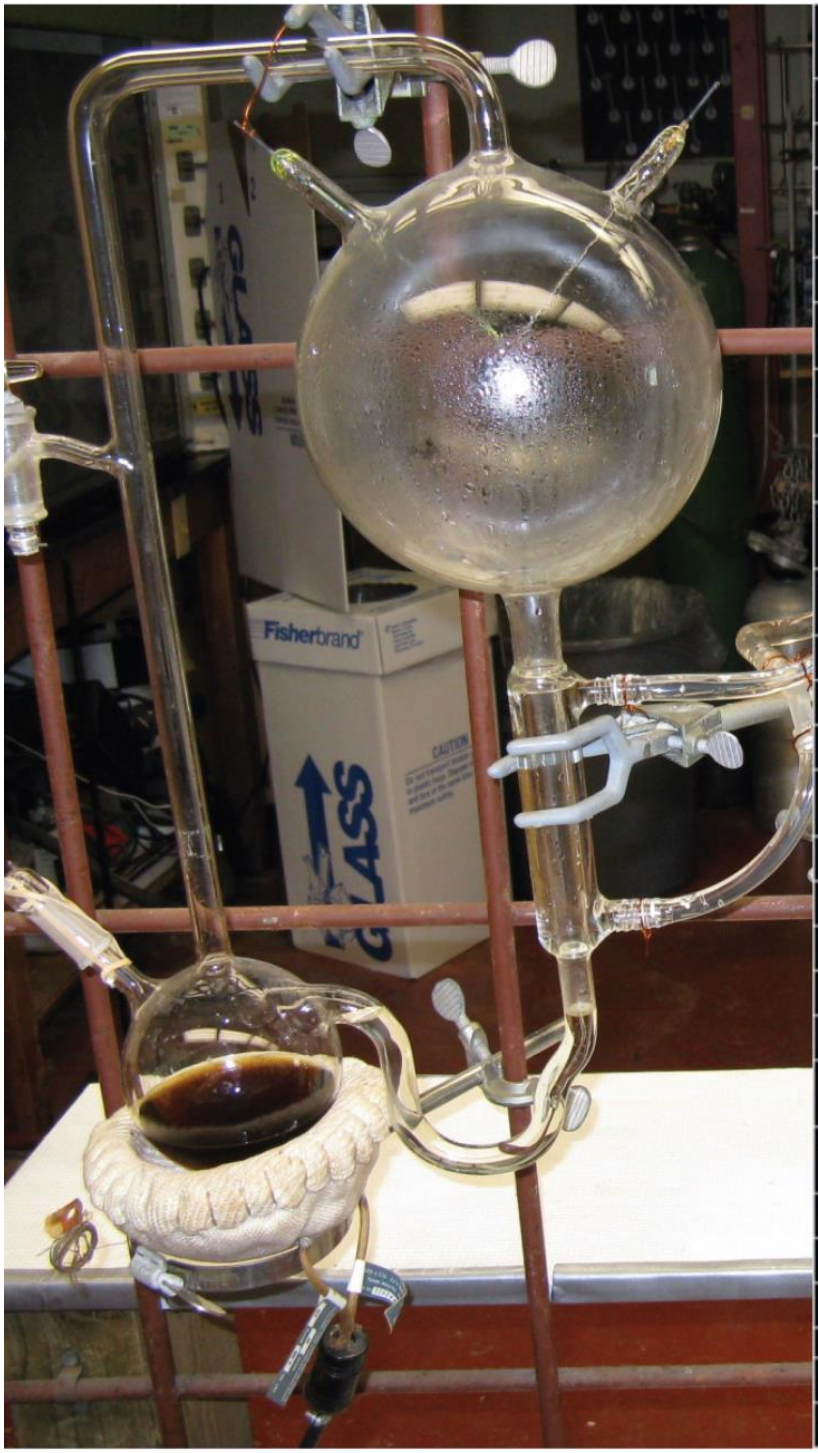
In March of 2007, a remarkable discovery was made, original samples from Miller's 1950s ED experiments that had been stored but not studied in depth. Furthermore, Miller labeled these samples with references to the exact laboratory notebook and page where more details could be found on the origins of his samples. Miller kept exceedingly diligent notes in his laboratory notebooks, explicitly stating the reagents used, how experiments were set up, and what (if any) analyses were completed. Thanks to Miller's foresight and careful note-taking, an incredible opportunity presented itself: to analyze the same samples produced by the pioneer in abiotic synthesis. However, today we have one very clear advantage over Miller, and that is the benefit of over 50 years of analytical instrumentation evolution. Current technologies can detect over 2-3 orders of magnitude more compounds than Miller could have, while being millions of times more sensitive than the techniques Miller had access to.

Some of Miller's archived samples (Figure 1.13) were previously analyzed, while some were unreported. Included in the haul were samples from Miller's classic-, volcanic-, and silent-, ED experiments, all of which were previously reported. Additional samples from an unreported experiment Miller conducted in which he simulated a H_2S-rich early Earth atmosphere, were also discovered.

Figure 1.13 Picture of original Miller spark discharge samples discovered in 2007. (Image Credit: Eric Parker)

1.7.1 *Re-analysis of the Classic MU Experiment*

Miller originally reported the detection of 5 AAs, and two unidentified species in his classic experiment. Johnson et al.[66] reported the re-analysis of original samples from Miller's classic ED experiment using HPLC-FD/TOF-MS, based on the methods developed by Glavin et al.[67] The analysis revealed that 14 AAs and 5 amines were produced by the experiment (Figure 1.14), but Miller could only identify a fraction of these compounds. In addition to the 14 AAs, numerous C_6 AAs were also detected, but were not quantified by this study.



	Apparatus One
Glycine (Gly)	1
Alanine (Ala)	2.7×10^{-2}
β-Alanine (β-Ala)	0.003
Serine (Ser)	1.0×10^{-4}
Isoserine (IsoSer)	Not detected
α-Amino-Isobutyric Acid (α-AIB)	1.1×10^{-3}
β-Amino-Isobutyric Acid (β-AIB)	3.2×10^{-5}
α-Amino-Butyric Acid (α-ABA)	7.0×10^{-4}
β-Amino-Butyric Acid (β-ABA)	5.0×10^{-4}
γ-Amino-Butyric Acid (γ-ABA)	1.0×10^{-4}
HomoSerine (HomoSer)	Not detected
2-Methyl Serine (2-Me-Ser)	Not detected
Aspartic Acid (Asp)	6.0×10^{-4}
β-hydroxy-Aspartic Acid (β-OH-Asp)	Not detected
Valine (Val)	3.3×10^{-5}
Isovaline (Isoval)	4.0×10^{-4}
Norvaline (Norval)	5.4×10^{-5}
Ornithine (Orn)	Not detected
Glutamic Acid (Glu)	2.0×10^{-4}
2-Methyl Glutamic Acid (2-Me-Glu)	Not detected
α-Amino Adipic Acid (α-AAA)	Not detected
Phenylalanine (Phe)	Not detected
Methylamine (MA)	5.0×10^{-3}
Ethylamine (EA)	1.7×10^{-3}
Ethanolamine	2.5×10^{-4}
Isopropylamine (Iso-PA)	1.3×10^{-5}
n-Propylamine (N-PA)	3.8×10^{-6}

Figure 1.14 Apparatus One (left), and the AAs and amines (right) produced by Miller during the execution of his classic experiment, as revealed by a re-analysis of Miller's original samples. Tabulated species were adapted, with permission, from Johnson et al. (2008) *Science*, 322, 404. (Image Credit: Eric Parker)

These archived samples were stored as dried residues at room temperature, in sealed, sterilized reaction vials. Due to the time that had passed from when the samples were produced and when they were re-analyzed over 50 years later, the question loomed as to whether or not storage conditions could have contaminated the samples. To address the issue of contamination, Johnson et al.[66] performed an analysis of the enantiomers of AAs with chiral centers using OPA/NAC pre-column derivatization, and determined that AA D/L ratios were close to 1 within experimental error. Additionally, a number of non-protein AAs, which are rare or non-existent in biology, were also detected. These combined pieces of evidence suggest that the detected species were products of the experiment, as opposed to being products of contamination during storage.

1.7.2 *Re-analysis of the Miller Volcanic Spark Discharge Experiment*

While Miller's classic experiment has been repeated and modified numerous times, his volcanic experiment gained considerably less fanfare. As a result, there was particular interest in re-analyzing Miller's original volcanic spark discharge samples because this *type* of ED experiment had never before been re-investigated. Johnson et al.[66] revisited the original samples from Miller's volcanic spark discharge experiment using OPA/NAC derivatization and HPLC-FD/TOF-MS analysis. Although Miller's reporting of the analysis of the volcanic experiment only detailed the detection and quantification of 5 AAs,[37] the re-analysis executed by Johnson et al.[66] using more sophisticated analytical techniques revealed that, in fact, 22 AAs and 5 amines were produced by the experiment (Figure 1.15). Therefore, all of these compounds were formed by the experiment Miller performed in the early 1950s, yet Miller was only aware of a handful of them because the analytical techniques available to Miller in the 1950s

were relatively crude and insensitive, thus demonstrating the benefit of analytical development to origin of life studies. These findings suggest that primitive environmental scenarios where a volcanic eruption coincides with lightning-rich air could have been a very powerful mechanism for the synthesis of biomolecules under abiotic conditions.

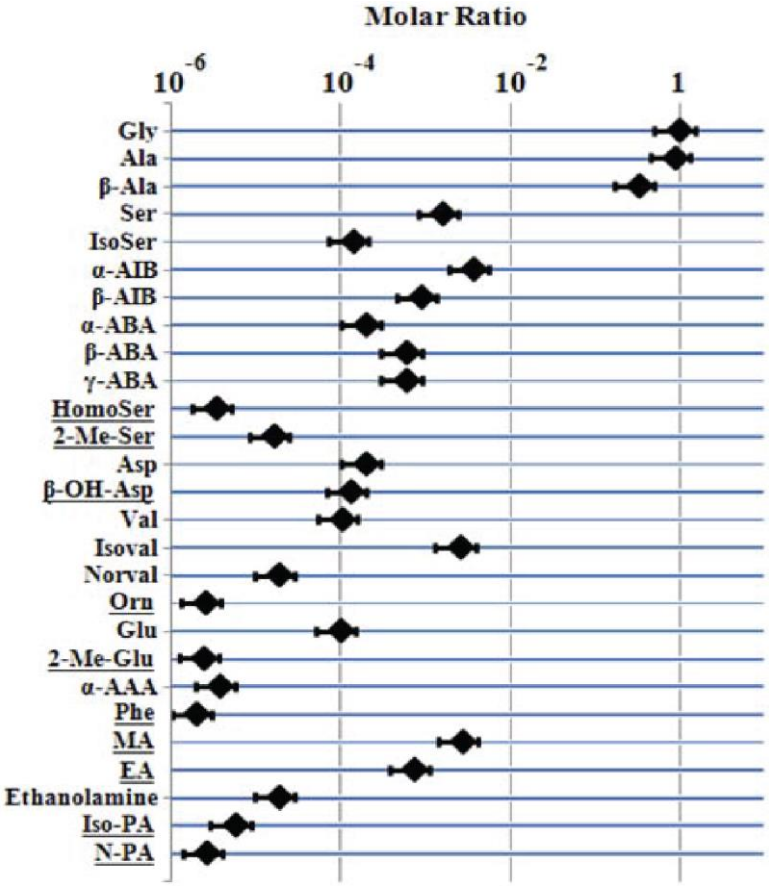


Figure 1.15 Molar ratio (relative to glycine = 1) of AAs and amines detected in Miller's original volcanic spark discharge experimental samples, via HPLC-FD/TOF-MS analysis. Figure is adapted, with permission, from Johnson et al. (2008) *Science*, 322, 404.

1.7.3 *Re-analysis of the Silent Electric Discharge Experiment*

In contrast to Miller's original analysis of the silent ED experiment where he only reported the detection of 5 AAs,[37] the re-analysis of the original samples from Miller's

silent discharge experiment resulted in the detection of 11 AAs and 1 amine (Figure 1.16), more than double the number of compounds originally detected by Miller.[66] In the re-analysis of these samples, sarcosine was not detected because Johnson et al.[66] used OPA/NAC derivatization, which targets primary amino groups, and sarcosine is a secondary AA.

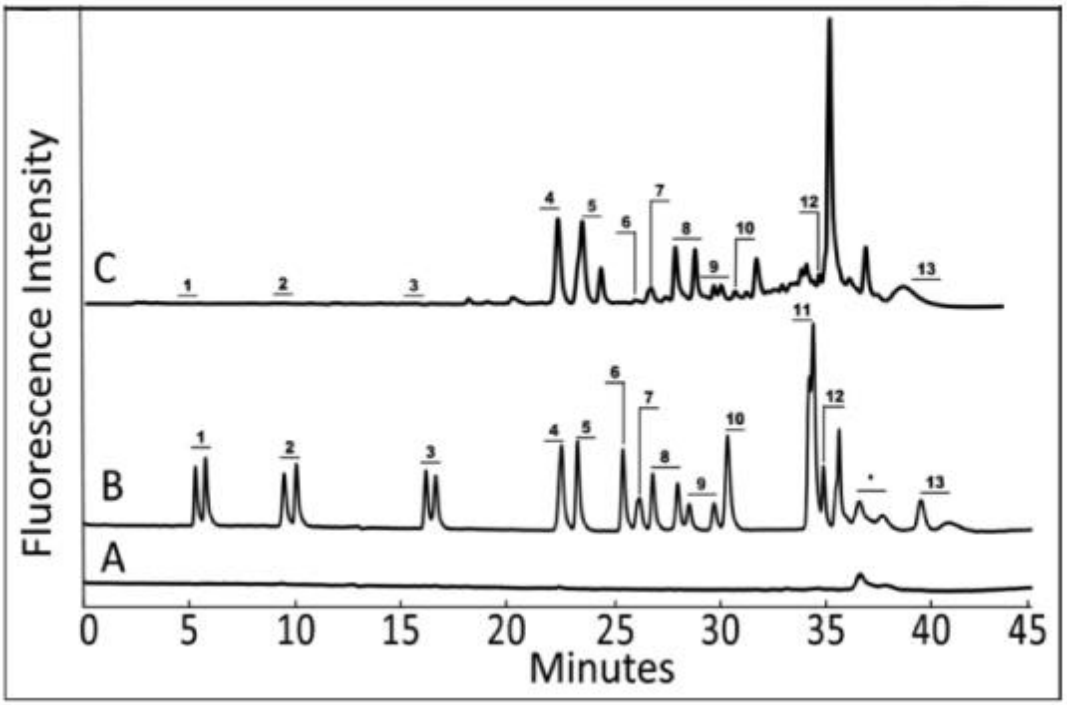


Figure 1.16 FD traces for A) an analytical blank, B) an AA standard, and C) the silent ED experiment. Peak identifications: 1 = D,L-Asp; 2 = L,D-Glu; 3 = D,L-Ser; 4 = Gly; 5 = β-Ala; 6 = γ-ABA; 7 = D,L-β-AIB; 8 = D,L-Ala; 9 = D,L-β-ABA; 10 = α-AIB; 11 = D,L-α-ABA, 12 = D,L-Iva; 13 = D,L-Nle. Here, trace C) is a summed chromatogram from multiple fractions. Peak heights and areas in trace C) are only intended to demonstrate the diversity of species produced in the silent experiment, and are not intended to represent quantitative abundances. Note: asterisks represent unidentified peaks. Figure is adapted, with permission, from Johnson et al. (2008) *Science*, 322, 404.

1.7.4 *The Hydrogen Sulfide Experiment*

By the mid-1950s, Miller had tested volcanic and non-volcanic conditions, as well as lightning-, and non-lightning-, based energy sources. Furthermore, Miller evaluated atmospheres with carbon-, hydrogen-, oxygen-, and nitrogen-, based species. But one aspect of his experiments was clearly lacking, and that was the inclusion of sulfur. Sulfur has significant ramifications for chemical evolution and the emergence of biology. For example, the sulfur AAs methionine and cysteine are important protein building blocks. Methionine facilitates the peptide chain elongation step of eukaryotic protein synthesis,[68] and cysteine promotes disulfide bond formation in eukaryotic proteins, contributing to protein folding.[69]

Miller's previous efforts did not address a major uncertainty regarding an early Earth environment where H_2 would have been available to reduce sulfur into H_2S, a plausible prebiotic gas due to current,[33] and likely ancient,[70] volcanic emissions. Furthermore, H_2S could have accumulated to become the dominant form of reduced sulfur on the early Earth.[71]

Extracts from the previously unreported 1958 Miller experiment that mimicked an H_2S-rich atmosphere were recently analyzed.[72] Miller used a version of his original apparatus and sparked CH_4, NH_3, H_2S, CO_2, and H_2O for 7 days before collecting and storing the samples. It remains a mystery why these samples went unreported. Parker et al.[72] used HPLC-FD and UPLC-FD/TOF-MS to detect 23 AAs and 4 amines, including 7 organosulfur compounds. An example of AA and amine detection by HPLC-FD is shown in Figure 1.17.

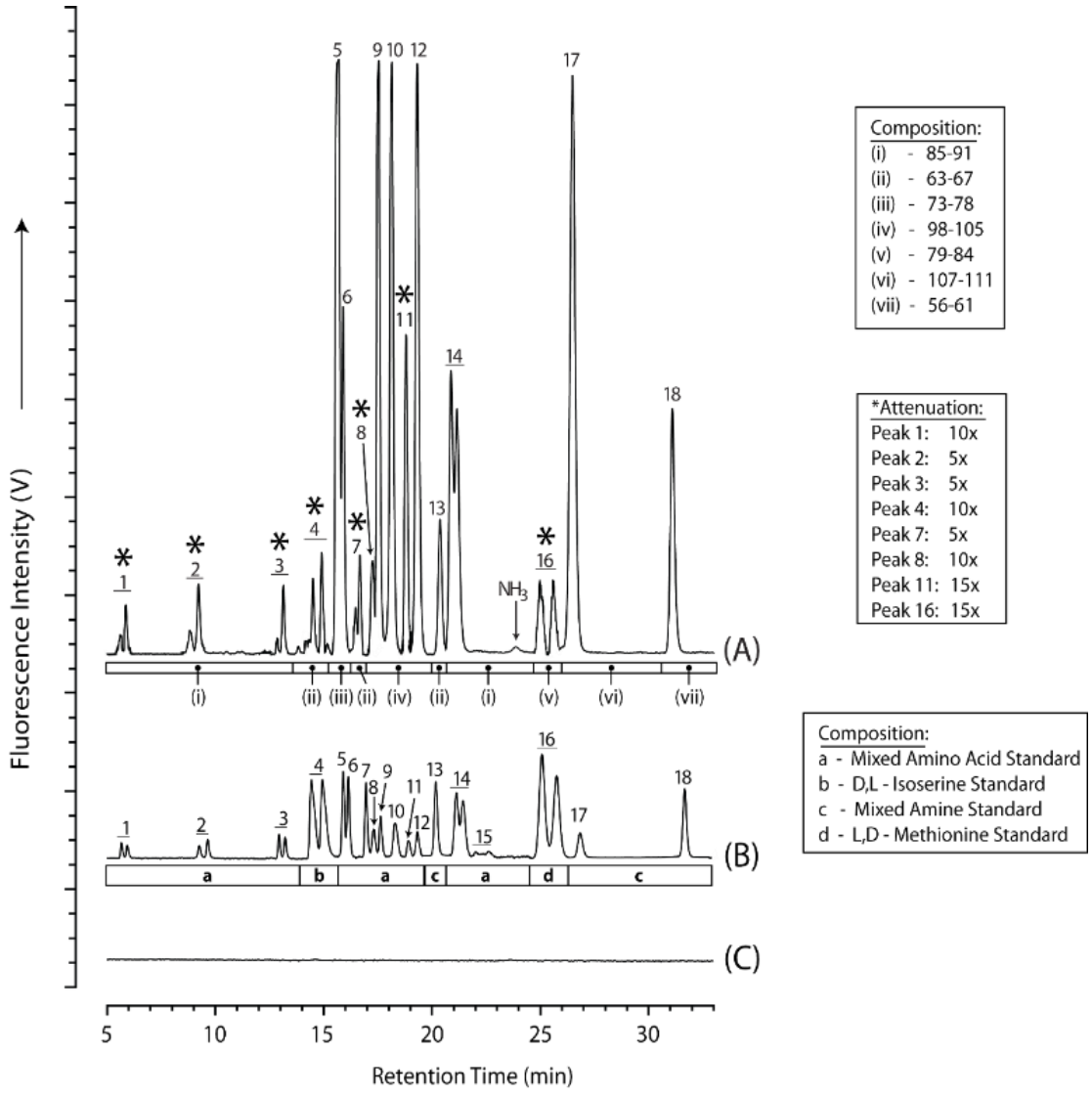


Figure 1.17 5-33 min HPLC-FD chromatograms of (A) Miller's original H_2S ED samples (B) a composite AA and amine standard, and (C) a reagent blank. Peak identifications: 1 = D,L-Asp; 2 = L,D-Glu; 3 = D,L-Ser; 4 = D,L-Ise; 5 = Gly; 6 = β-Ala; 7 = γ-ABA; 8 = β-AIB; 9 = D-Ala; 10 = L-Ala + D-β-ABA; 11 = L-β-ABA; 12 = α-AIB; 13 = ethanolamine; 14 = D,L-α-ABA; 15 = D,L-Iva; 16 = L,D-Met; 17 = methylamine; 18 = ethylamine. Figure and figure legend are adapted, with permission, from Parker et al. (2011) *Proceedings of the National Academy of Sciences U.S.A.*, 108, 5526-5531.

Of the 7 organosulfur compounds formed, 6 were AAs and 1 was an amine.[73] Methionine, and its oxidation products, methionine sulfoxide and methionine sulfone were among the AAs detected. Methionine's oxidation products were likely a result of

sample storage under non-anoxic conditions, and are indicative that methionine probably was not stable after the onset of the great oxidation event ~2.5 bya. Parker et al.[73] proposed formation mechanisms for numerous organosulfur species, in addition to demonstrating analytical detections (Figure 1.18).

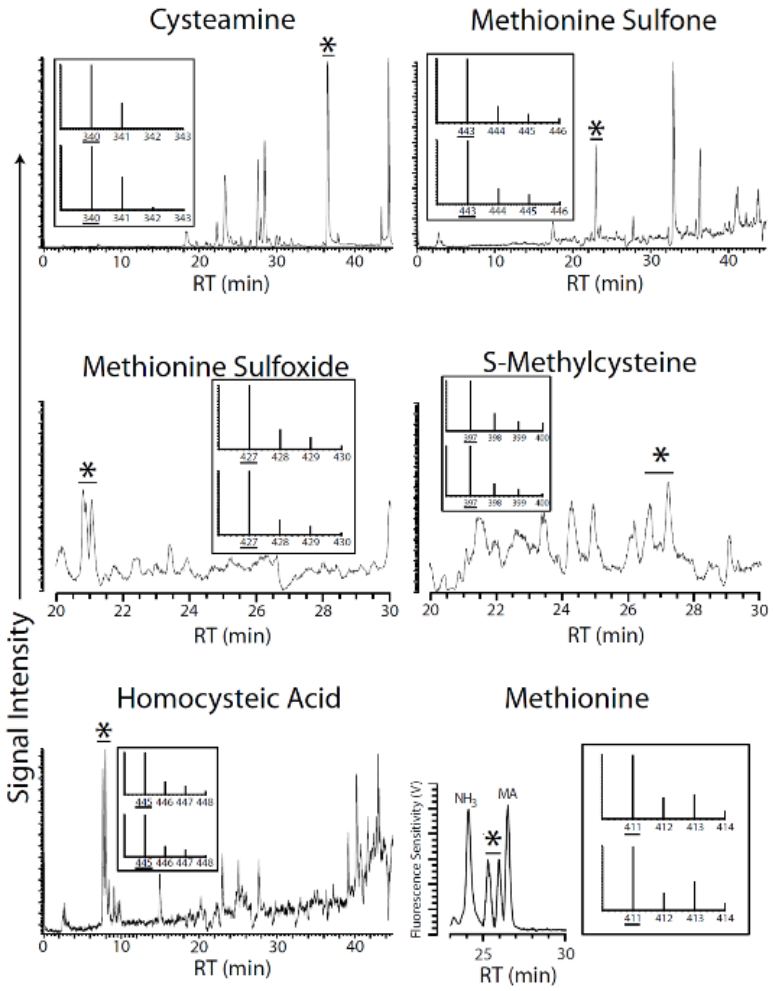


Figure 1.18 Sample chromatograms and mass spectral traces for 6 sulfur compounds detected in Miller's original sample extracts. All chromatogram traces resulted from selective ion monitoring analysis (signal intensity vs. time) except the methionine trace that was produced by HPLC-FD analysis (fluorescence intensity vs. time). Asterisks demarcate the detection of the species in question. Mass spectral traces were obtained using ToF-MS, and are plotted as spectral intensity versus mass-to-charge. In all cases, the bottom mass spectral traces are the standard traces and the top mass spectral traces are the experimental traces. In each mass spectral trace, the underlined mass is the parent mass. Note: RT is retention time and MA is methylamine. Figure and figure legend are adapted, with permission, from Parker et al. (2011) *Origins of Life and Evolution of Biospheres*, 41, 201-212.

Parker et al.[74] further discovered that Miller's H_2S experiment produced an unusually high proportion of alkyl AAs. The species of interest included the α-, β-, and γ-isomers of aminobutyric acid. The findings reported that alkyl AAs can be synthesized from aspartic and glutamic acids in the presence of H_2S via the formation of thiomide intermediates (Figure 1.19). It was also reported that H_2S gas, when in the presence of an ED, is a source of reactive sulfur radicals that can initiate the synthesis of AA precursors, such as aldehydes and ketones.

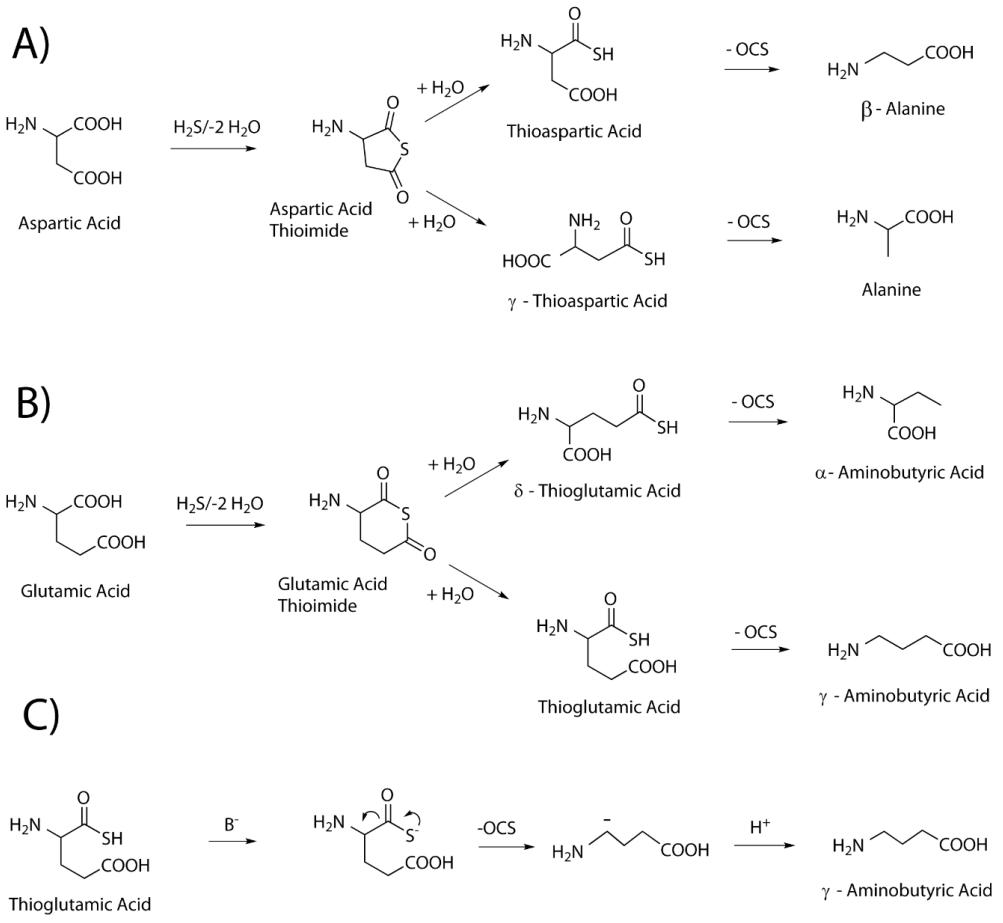


Figure 1.19 Proposed mechanisms for the aqueous phase production of alkyl AAs from A) aspartic acid and B) glutamic acid, in the presence of H_2S. A more detailed mechanism starting from thioglutamic acid is shown in C). Figure and figure legend are adapted, with permission, from Parker et al. (2011) *Origins of Life and Evolution of Biospheres*, 41, 569-574.

A comparison of results from Miller's H_2S experiment with those of the Murchison and LON 94102 meteorites, provides intriguing astrochemical implications. Murchison was a ~100 kg meteorite[75] that fell in Murchison, Australia in 1969, and has been extensively studied for extraterrestrial biomolecules.[76-78] Miller and colleagues conducted ED experiments in which all the α-AAs and non-protein AAs that were presently known to be in the Murchison meteorite, were synthesized,[79, 80] demonstrating that MU ED reactions represent primitive conditions. A striking resemblance in AA profiles exists between Miller's H_2S experiment and the Murchison and LON 94102 meteorites (Figure 1.20), suggesting that H_2S may have influenced abiotic synthesis on Earth, and elsewhere in the solar system.

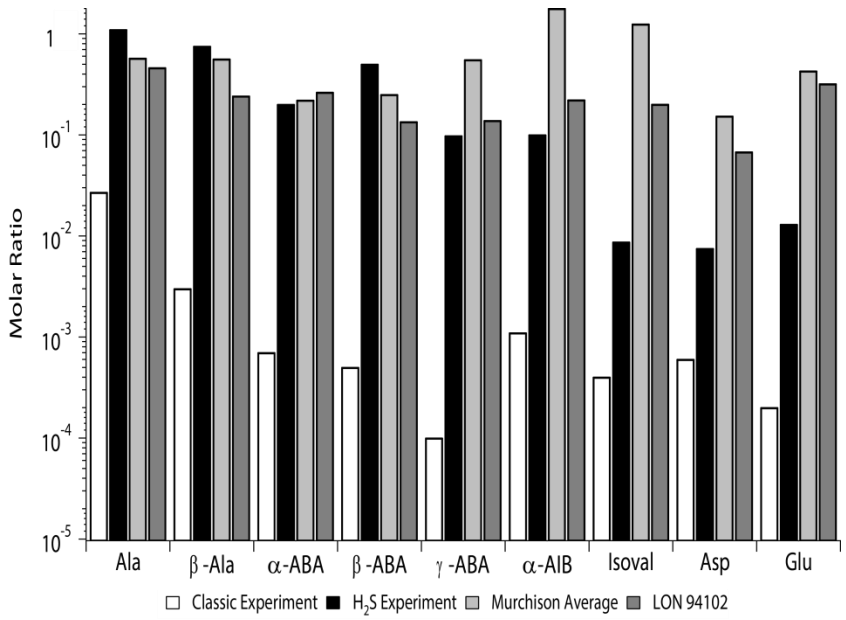


Figure 1.20. Comparison of AA molar ratios (relative to glycine = 1) found in Miller's H_2S and classic spark discharge experiments, and the Murchison USNM 5453, Murchison USNM 6650, and the LON 94102 meteorites. Data from the two Murchison meteorite samples were averaged and are presented here as "Murchison Average". The classic ED data were reported in Johnson et al.[66] Literature data were used for the Murchison USNM 5453,[81] Murchison USNM 6650,[63, 67] and LON 94102[63, 81] samples. Figure and figure legend are adapted, with permission, from Parker et al. (2011) *Proceedings of the National Academy of Sciences U.S.A.*, 108, 5526-5531.

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CHAPTER 2: CONDUCTING MILLER-UREY EXPERIMENTS

Adapted with permission from:
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2.1 Abstract

Subsequent to Stanley Miller's publishing the synthesis of AAs from the simulation of a primitive ocean-atmosphere system via conducting the classic MU experiment, there has been much interest in further testing possible early Earth conditions to evaluate the formation of molecules important for life. Performing prebiotic simulation experiments, particularly of the MU ED variety, continues to be of interest to the origin of life and prebiotic chemistry communities. To illustrate, conducting such experiments was of paramount importance for the execution of the research discussed throughout this thesis. However, the steps necessary to execute these experiments were previously unreported in detail. Consequently, a lack of knowledge existed in the origin of life and prebiotic chemistry fields, with respect to properly conducting ED experiments. Such a gaping void in the literature illustrated that the two fields would most certainly benefit from the reporting of the methods used to conduct these prebiotic experiments correctly and safely. The purpose of this chapter is to provide the first, established, working experimental protocol for the execution of MU ED experiments, using a simplified 3 L reaction flask. Since the experiments involve exposing inflammable gases to a high voltage ED, it is worth highlighting important steps that reduce the risk of explosion. The establishing of the protocol outlined in this chapter was crucial to the completion of this

thesis, and can be extrapolated to design and conduct a wide variety of ED experiments simulating primitive planetary environments.

2.2 Introduction

In the 1950s, chemists began to conduct targeted laboratory studies aimed at understanding how organic molecules could have been synthesized from simple starting materials on the early Earth. One of the first reports to this end was the synthesis of formic acid from the irradiation of aqueous CO_2 solutions in 1951.[1] However, the groundbreaking work of Stanley Miller,[2] using his classic apparatus (Figure 2.1.A), is considered to be the first deliberate, efficient synthesis of biomolecules under simulated primitive Earth conditions. Specific details about the apparatus Miller used can be found elsewhere.[3]

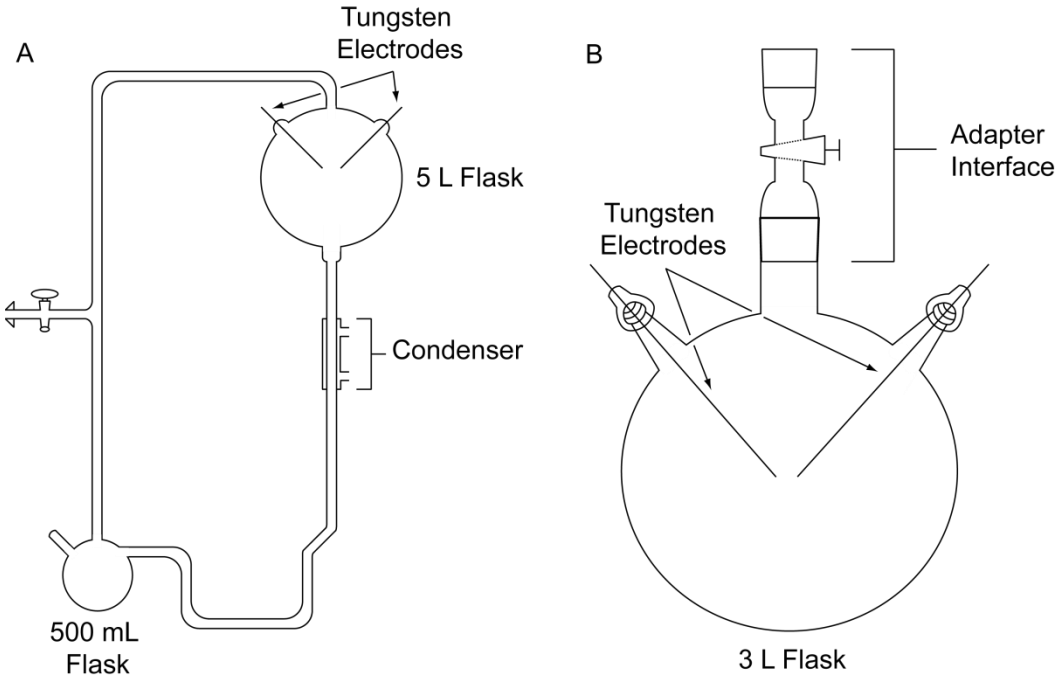

Figure 2.1 Comparison between the two types of apparatuses discussed in this chapter. The classic apparatus used for the original MU experiment (A) and the simplified apparatus used in the protocol outlined here (B).

After the 1953 publication of results from Miller's classic experiment, numerous variations of the spark discharge experiment, for example using other gas mixtures, were performed to explore the plausibility of producing organic compounds important for life under a variety of possible early Earth conditions. For example, a $CH_4/H_2O/NH_3/H_2S$ gas mixture was tested for its ability to produce the coded sulfur-containing α-AAs, although these were not detected.[4] Gas chromatography-mass spectrometry (GC-MS) analysis of a CH_4/NH_3 mixture subjected to an ED showed the synthesis of α-aminonitriles, which are AA precursors.[5] In 1972, using a simpler apparatus, first introduced by Oró[6] (Figure 2.1.B), Miller and colleagues demonstrated the synthesis of all of the coded α-AAs[7] and non-protein AAs[8] that had been identified in the Murchison meteorite to date, by subjecting CH_4, N_2, and small amounts of NH_3 to an ed. Later, using this same simplified experimental design, gas mixtures containing H_2O, N_2, and CH_4, CO_2, or CO were sparked to study the yield of HCN, formaldehyde, and AAs as a function of the oxidation state of atmospheric carbon species.[9]

In addition to the exploration of alternative experimental designs, significant analytical advances have occurred since Miller's classic experiment, which recently aided more probing investigations of ED samples archived by Miller, than would have been facilitated by the techniques Miller had access to in the 1950s. Miller's volcanic experiment,[10] first reported in 1955,[3] and a 1958 H_2S-containing experiment[11] were shown to have formed a wider variety, and greater abundances, of numerous AAs and amines than the classic experiment, including many of which that had not been previously identified in spark discharge experiments.

The experiment described in this chapter can be conducted using a variety of gas mixtures. Typically, at the very least, such experiments will contain a C-bearing gas, an N-bearing gas, and water. With some planning, almost any mixture of gases can be explored, however, it is important to consider some chemical aspects of the system. For example, the pH of the aqueous phase can have a significant impact on the chemistry that occurs there.[12]

The method described here is tailored to instruct researchers how to conduct spark discharge experiments that resemble the MU experiment using a simplified 3 L reaction vessel, as described in Miller's 1972 publications.[7, 8] Since this experiment involves a high voltage electric arc acting on inflammable gases, it is ***crucial*** to remove O_2 from the reaction flask to eliminate the risk of explosion, which can occur upon combustion of reduced carbon-bearing gases such as CH_4 or CO, or reaction of H_2 with O_2.

There are additional details that should be kept in mind when preparing to conduct the experiment discussed here. First, whenever working with glass vacuum lines and pressurized gases, there exists the inherent danger of both implosion and over-pressuring. ***Therefore, safety glasses must be worn at all times.*** Second, the experiment is typically conducted at less than atmospheric pressure. This minimizes the risk of over-pressuring the manifold and reaction flask. Glassware may be rated at or above atmospheric pressure, however, pressures above 1 atm are not recommended. Pressures may increase in these experiments as water-insoluble H_2 is liberated from reduced gases (such as CH_4 and NH_3). Over-pressuring can lead to seal leakage, which can allow atmospheric O_2 to enter the reaction flask, making it possible to induce combustion, resulting in an explosion. Third, it should be borne in mind that modification of this

protocol to conduct variations of the experiment requires careful planning to ensure unsafe conditions are not created. Fourth, it is highly recommended that the prospective experimenter read through the entire protocol ***carefully*** several times prior to attempting this experiment to be sure he or she is familiar with potential pitfalls and that all necessary hardware is available and in place. Lastly, conducting experiments involving combustible gases require compliance with the experimenter's host institution's Environmental Health and Safety departmental guidelines. Please observe these recommendations before proceeding with any experiments. All steps detailed in the protocol here are in compliance with the authors' host institutional Environmental Health and Safety guidelines.

2.3 Protocol

Note: The material used for this experiment, and associated pertinent information, will be tabulated in Section 2.6 at the end of this chapter, as the "List of Materials Used".

2.3.1 *Setting Up a Manifold/Vacuum System*

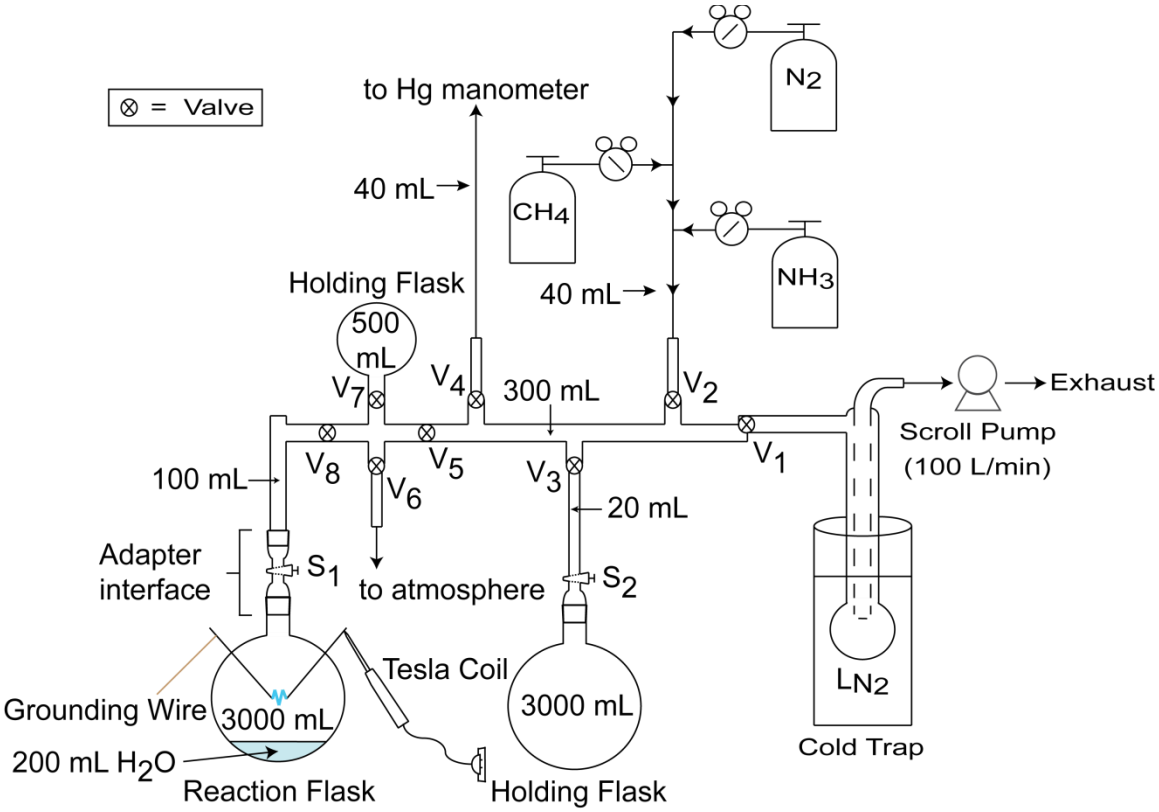

Figure 2.2. Manifold/vacuum system used to introduce gases into the 3 L reaction flask. Valves controlling gas flow are labeled as V_1 - V_8, while stopcocks controlling gas flow are labeled as S_1 and S_2. It is worth noting that while Valves 1, 2, and 6, and Stopcock 1 are referred to explicitly in the protocol, the other valves and stopcock in the manifold shown here are useful for adding or removing volume (*i.e.*, holding flasks) to or from the manifold. For example, when introducing gases into the manifold at relatively high pressures (approximately 500 mmHg or greater), it is advised that the experimenter makes use of all holding flasks attached to the manifold to increase the accessible volume of the manifold and help minimize the risk of over-pressuring the manifold.

2.3.1.1 Use a glass manifold for introduction of gases to the reaction flask. The manifold can be purchased or constructed by a glass-blowing facility, but must include vacuum-tight ports for connection to a vacuum system, vacuum gauge, reaction vessel, and gas cylinders.

2.3.1.2 Use ground glass joints and glass plugs with valves on the manifold. Ensure that all O-rings on the plugs are capable of making the necessary seals. If using glass joints, a sufficient amount of vacuum grease can be applied to help make a seal, if necessary. Silicon vacuum grease can be used to avoid potential organic contamination.

2.3.1.3 Use glass stopcocks on the manifold. Apply the minimum amount of vacuum grease necessary to make a seal.

2.3.1.4 Measure the manifold volume. This volume will be used for calculations related to final gas pressures in the 3 L reaction flask and should be known as precisely as possible.

2.3.1.5 Unless the manifold has enough connections to accommodate all gas cylinders simultaneously, connect one cylinder at a time to the manifold. Include in this connection, a tap allowing the manifold to be isolated from the ambient atmosphere.

2.3.1.6 Use suitable, clean, inert, and chemical and leak resistant tubing and ultra-Torr vacuum fittings to connect the gas cylinders to the manifold. Ultra-Torr fittings, where used, are to be finger-tightened.

2.3.1.7 Connect to the manifold, a vacuum pump capable of establishing a vacuum of <1 mmHg. The vacuum pump exhaust should be located within the fume hood, or properly vented by other means.

2.3.1.7.1 To ensure rapid attainment of vacuum and to protect the pump, insert a trap between the manifold and vacuum pump. A liquid nitrogen finger-trap is recommended and will prevent volatiles such as NH_3, CO_2, and H_2O from entering the pump. Note: trapped volatiles, upon warming, may overpressure the manifold and result in glass rupture.

2.3.1.8 Connect to the manifold, a manometer or other vacuum gauge capable of 1 mmHg resolution or better. While various devices can be used, a mercury manometer, or MacLeod gauge, is preferable as mercury is fairly non-reactive.

2.3.1.9 Measure and record the ambient temperature using a suitable thermometer.

2.3.2 ***Preparation of Reaction Flask***

2.3.2.1 Heat all glassware at 500°C for at least 3 hours in air prior to use, to remove organic contaminants.

2.3.2.2 Clean the tungsten electrodes by gently washing with clean laboratory wipes and methanol, and drying in air.

2.3.2.3 Pour 200 mL ultrapure H_2O (18.2 MΩ cm, <5 ppb TOC) into 3 L reaction flask.

2.3.2.3.1 Introduce a pre-cleaned and sterilized magnetic stir bar, which will ensure rapid dissolution of soluble gases and mixing of reactants during the experiment.

2.3.2.4 Attach tungsten electrodes to the 3 L reaction flask using minimal vacuum grease, with tips separated by approximately 1 cm inside the flask. Fasten with clips.

2.3.2.5 Insert an adapter with a built-in stopcock into the neck of the 3 L reaction flask and secure with a clip.

2.3.2.6 Attach the 3 L reaction flask to the gas manifold via the adapter. Use a clip or clamp to help secure the flask.

2.3.2.6.1 Lightly grease all connections to ensure a good vacuum seal.

2.3.2.7 Open all valves and stopcocks on the manifold, except Valve 6 and Stopcock 1 (Figure 2.2), and turn on the vacuum pump to evacuate the manifold. Once a stable vacuum reading of <1 mmHg has been attained, close Valve 1 and allow the manifold to sit for ~ 15 minutes to check for vacuum leaks. If none are detected, proceed to step *2.3.2.8.* Otherwise troubleshoot the various connections until the leaks can be identified and fixed.

2.3.2.8 Apply magnetic stirring to reaction vessel. Open Valve 1 and Stopcock 1 (Figure 2.2) to evacuate the headspace of the 3 L reaction flask until the pressure <1 mmHg.

2.3.2.9 Close Valve 1 (Figure 2.2) and monitor the pressure inside the 3 L reaction flask. The measured pressure should increase to the vapor pressure of water. To ensure that no leaks exist, wait ~ 5 minutes at this stage. If the pressure (as read on the manometer) increases while Valve 1 is closed during this step, check for leaks in Stopcock 1 and the various reaction flask connections. If no leak is found, proceed to the next step.

2.3.3 *Introduction of Gaseous NH_3*

2.3.3.1 Calculate the necessary pressure of gaseous NH_3 to introduce into the manifold such that 200 mmHg of NH_3 will be introduced into the reaction flask. Details on how to do this are provided in the Discussion section of this chapter.

2.3.3.2 Close Valves 1 and 6, and Stopcock 1 (Figure 2.2) before introducing any gas into the manifold. Leave the other valves and stopcock open.

2.3.3.3 Introduce NH_3 into the manifold until a small pressure (~10 mmHg) is reached and then evacuate the manifold to a pressure of <1 mmHg by opening Valve 1 (Figure 2.2). *Repeat 3 times*.

2.3.3.4 Introduce NH_3 into manifold to reach the pressure obtained in step *2.3.3.1*.

2.3.3.5 Open Stopcock 1 (Figure 2.2) to introduce 200 mmHg of NH_3 into the 3 L reaction flask. The NH_3 will dissolve in the water in the reaction flask and the pressure read by the manometer, will fall slowly.

2.3.3.6 Once the pressure stops dropping, close Stopcock 1 (Figure 2.2) and record the pressure read by the manometer. This is the pressure inside the flask and will be used to calculate the pressures for other gases that will be introduced into the manifold later.

2.3.3.7 Open Valve 1 (Figure 2.2) to evacuate the manifold to a pressure of <1 mmHg.

2.3.3.8 Close Valve 2 (Figure 2.2) and disconnect the NH_3 cylinder from the manifold.

2.3.4 *Introduction of CH_4*

2.3.4.1 Calculate the necessary pressure of CH_4 to be introduced into the manifold such that 200 mmHg of CH_4 will be introduced into the 3 L reaction flask. Example calculations are shown in the Discussion section of this chapter.

2.3.4.2 Connect the CH_4 gas cylinder to the manifold.

2.3.4.3 Open all valves and stopcocks, except Valve 6 and Stopcock 1 (Figure 2.2), and evacuate the manifold to a pressure of <1 mmHg.

2.3.4.4 Close Valve 1 once the manifold has been evacuated (Figure 2.2).

2.3.4.5 Introduce CH_4 into the manifold until a small pressure (~10 mmHg) is obtained. This purges the line of any contaminant gases from preceding steps. Open Valve 1 (Figure 2.2) to evacuate the manifold to <1 mmHg. *Repeat twice more.*

2.3.4.6 Introduce CH_4 into the manifold to reach the pressure from step *2.3.4.1*.

2.3.4.7 Open Stopcock 1 (Figure 2.2), introduce 200 mmHg CH_4 in the reaction flask.

2.3.4.8 Close Stopcock 1 once the intended pressure of CH_4 has been introduced into the 3 L reaction flask (Figure 2.2) and record the pressure measured by the manometer.

2.3.4.9 Open Valve 1 (Figure 2.2) to evacuate the manifold to <1 mmHg.

2.3.4.10 Close Valve 2 (Figure 2.2) and disconnect CH_4 from the manifold.

2.3.5 *Introduction of Further Gases (e.g., N_2)*

2.3.5.1 At this point, it is not necessary to introduce additional gases. However, if desired, it is recommended to add 100 mmHg of N_2. In this case, calculate the necessary pressure of N_2 to be introduced into the manifold such that 100 mmHg of N_2 will be introduced into the 3 L reaction flask. Example calculations are shown in the Discussion section of this chapter.

2.3.5.2 Connect the N_2 gas cylinder to the manifold.

2.3.5.3 Open all valves and stopcocks, except Valve 6 and Stopcock 1 (Figure 2.2), and evacuate the manifold to a pressure of <1 mmHg.

2.3.5.4 Close Valve 1 once the manifold has been evacuated (Figure 2.2).

2.3.5.5 Introduce N_2 into the manifold until a small pressure (~10 mmHg) is obtained. Open Valve 1 (Figure 2.2) to evacuate the manifold to <1 mmHg. *Repeat twice more.*

2.3.5.6 Introduce N_2 into the manifold to reach the pressure from step *2.3.5.1*.

2.3.5.7 Open Stopcock 1 (Figure 2.2) to introduce 100 mmHg N_2 into the reaction flask.

2.3.5.8 Close Stopcock 1 once the intended pressure of N_2 has been introduced into the reaction flask (Figure 2.2), and record the pressure using the manometer.

2.3.5.9 Open Valve 1 (Figure 2.2) to evacuate the manifold to <1 mmHg.

2.3.5.10 Close Valve 2 (Figure 2.2) and disconnect the N_2 cylinder from the manifold.

2.3.6 *Beginning the Experiment*

2.3.6.1 Detach the reaction flask from the manifold by closing Stopcock 1 and Valve 1 (Figure 2.2) once all gases have been introduced into the reaction flask, so that ambient air may enter the manifold and bring the manifold up to ambient pressure.

2.3.6.2 After carefully disconnecting the reaction flask from the manifold, set the flask somewhere it will not be disturbed (e.g., inside an empty fume hood).

2.3.6.3 Disconnect the vacuum pump and carefully remove the cold trap and allow venting inside a fully operational fume hood.

2.3.6.4 Secure the Tesla coil connected to the high frequency spark generator.

2.3.6.5 Connect the opposite tungsten electrode to an electrical ground to enable the efficient passage of electrical current across the gap between the two electrodes.

2.3.6.6 Set the output voltage of the spark generator to approximately 30,000 V, as detailed by documents available from the manufacturer.

2.3.6.7 Prior to initiating the spark, close the fume hood sash, to serve as a safety shield between the apparatus and the experimenter. Turn the Tesla coil on to start the experiment, and allow sparking to continue for 2 weeks (or other desired period) in 1 hour on/off cycles.

2.3.7 *End of Experiment*

2.3.7.1 Stop the experiment by turning off the Tesla coil.

2.3.7.2 Slowly open Stopcock 1 (Figure 2.2) to introduce ambient air in the reaction flask, and aid removal of the adapter and tungsten electrodes prior to sample collection.

2.3.8 *Collecting Liquid Sample*

2.3.8.1 Using a pyrolyzed glass pipette, remove liquid samples from the reaction flask, being careful to minimize exposure to contaminants, such as those that might be introduced by touching the pipette to the vacuum grease or other non-sterile surfaces.

2.3.8.2 Transfer the sample to a sterile plastic or glass receptacle. Plastic receptacles are less prone to cracking or breaking upon freezing, compared to glass receptacles.

2.3.8.3 Seal sample containers and store in a freezer capable of reaching temperatures of -20°C or lower. Insoluble products may prevent sample solution from freezing at 0 °C.

2.3.9 *Cleaning the Apparatus*

2.3.9.1 Use clean laboratory wipes to carefully remove vacuum grease from the neck of the apparatus, the adapter and stopcock, and the glass surrounding the electrodes.

2.3.9.2 Thoroughly clean the surfaces described in step *2.3.9.1.* with toluene to remove vacuum grease. If using silicon grease, the grease may remain on the glassware after pyrolysis, creating future problems, as detailed in the Discussion section of this chapter.

2.3.9.3 Clean reaction flask with brush and the following solvents in order: ultrapure water (18.2 MΩ cm, <5 ppb TOC), ultrapure water (18.2 MΩ cm, <5 ppb TOC) with 5% cleaning detergent, methanol, toluene, methanol, ultrapure water (18.2 MΩ cm, <5 ppb TOC) with 5% cleaning detergent, and ultrapure water (18.2 MΩ cm, <5 ppb TOC).

2.3.9.4 Cover all open orifices of the reaction flask with aluminum foil and wrap the adapter and its components in aluminum foil.

2.3.9.5 Once all the glassware has been wrapped in aluminum foil, pyrolyze for at least 3 hours in air at 500°C.

2.3.9.6 Gently clean electrodes with methanol and let air dry.

2.3.10 *Sample Analysis*

Note: When preparing samples for analysis, the use of an acid hydrolysis protocol such as has been described in detail elsewhere,[13] is useful for obtaining more AAs. Hydrolysis of a portion of the recovered sample provides the opportunity to analyze both free AAs as well as their acid-labile precursors that are synthesized under abiotic conditions.

2.3.10.1 For AA analysis, use a suitable technique (such as liquid chromatography-, and mass spectrometry-based methods, or other appropriate approaches). Such analytical techniques include HPLC-FD,[12] and UPLC with FD in parallel with TOF positive ESI mass spectrometry.[10, 11] This manuscript describes analysis using mass spectrometric analyses via triple quadrupole mass spectrometry (QqQ-MS) with HPLC-FD.

2.4 Representative Results

The products synthesized in ED experiments can be complex, and numerous analytical approaches can be used to study them. Some of the more commonly used techniques in the literature for analyzing AAs are discussed here. Chromatographic and mass spectrometric methods are highly informative techniques for analyzing the complex chemical mixtures produced by MU type spark discharge experiments. AA analyses can be conducted using OPA/NAC,[14] yielding fluorescent diastereomer derivatives that can be separated on an achiral stationary phase. Figure 2.3 shows a chromatogram of an OPA/NAC-derivatized AA standard obtained by HPLC coupled to FD and QqQ-MS. The AAs contained in the standard include those typically produced in MU spark discharge experiments. The identities of these AAs are listed in Table 2.1. Representative fluorescence traces of a typical sample and analytical blank are shown in Figure 2.4, demonstrating the complexity of MU ED samples. The sample chromatogram in Figure 2.4 was produced from a spark discharge experiment using the following starting conditions: 300 mmHg of CH_4, 250 mmHg of NH_3 and 250 mL of water.

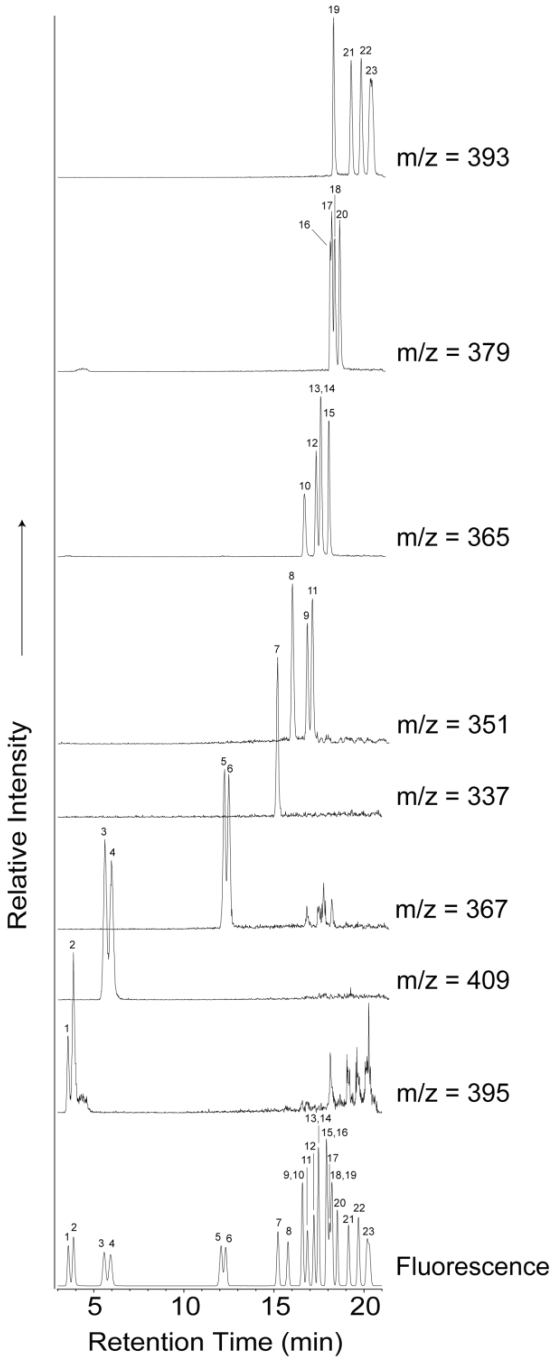

Figure 2.3 The 3-21 min region of the HPLC-FD/QqQ-MS chromatograms produced from the analysis of an OPA/NAC-derivatized AA standard. AA peak identities are listed in Table 1. The fluorescence trace is shown at the bottom and the corresponding extracted mass chromatograms are shown above. The ESI QqQ-MS was operated in positive ion mode and monitored a mass-to-charge (m/z) range of 50-500 m/z. The ESI settings were: desolvation gas (N_2) temperature: 350°C, 650 L/hr; capillary voltage: 3.8 kV; cone voltage: 30 V. The unlabeled peaks in the 367 extracted ion chromatogram are the $^{13}C_2$ peaks from the 365 extracted ion chromatogram, as a result of the approximately 1% natural abundance of ^{13}C.

Table 2.1 Peak identities for AAs detected in the standard and that are typically produced in MU spark discharge experiments.

Peak	AA
1	D-aspartic acid
2	L-aspartic acid
3	L-glutamic acid
4	D-glutamic acid
5	D-serine
6	L-serine
7	Glycine
8	β-Alanine
9	D-alanine
10	γ-amino-*n*-butyric acid (γ-ABA)
11	L-alanine
12	D-β-amino-*n*-butyric acid (D-β-ABA)
13	α-aminoisobutyric acid (α-AIB)
14	L-β-amino-*n*-butyric acid (L-β-ABA)
15	D,L-α-amino-*n*-butyric acid (D,L-α-ABA)
16	D-isovaline
17	L-isovaline
18	L-valine
19	ε-amino-*n*-caproic acid (EACA)
20	D-valine
21	D-isoleucine
22	L-isoleucine
23	D,L-leucine

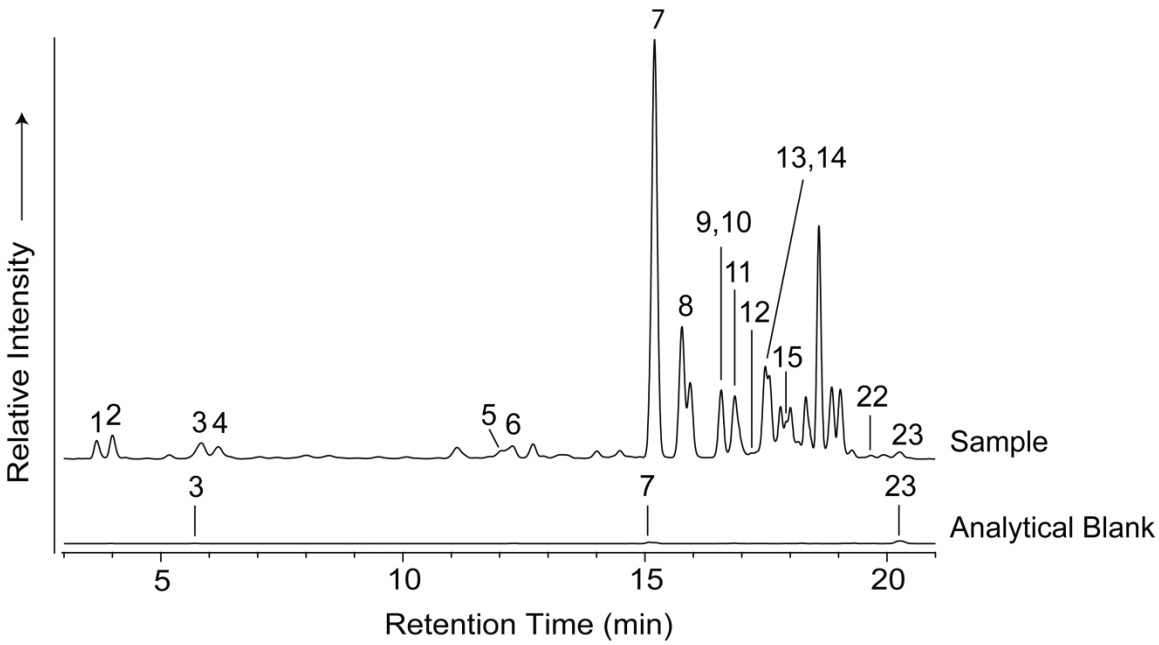

Figure 2.4 The 3-21 min region of the HPLC-FD chromatograms representative of MU ED experiments. Peaks were identified by retention time and mass analysis of target compounds, and quantitated by comparison to a standard and analytical blank. All target analytes with co-eluting fluorescence retention times can be separated and quantitated using mass spectrometry, except for α-AIB and L-β-ABA (peaks 13 and 14), and D/L-norleucine, which co-elutes with D/L-leucine (peak 23), under the chromatographic conditions used. D/L-norleucine was added as an internal standard to samples and analytical blanks during sample preparation. AAs were separated using a 4.6 mm x 250 mm, 5 μm particle size Phenyl-Hexyl HPLC column. The mobile phase included: A) ultrapure water (18.2 MΩ cm, <5 ppb TOC), B) methanol, and C) 50 mM ammonium formate (NH_4Fo) with 8% methanol, at pH 8. The gradient was: 0-5 min, 100% C; 5-15 min, 0-83% A, 0-12% B, 100-5% C; 15-22 min, 83-75% A, 12-20% B, 5% C; 22-35 min, 75-35% A, 20-60% B, 5% C; 35-37 min, 35-0% A, 60-100% B, 5-0% C; 37-45 min, 100% B; 45-46 min, 100-0% B, 0-100% C 46-55 min, 100% C. The flow rate was 1 mL/min.

2.5 Discussion

Numerous steps in the protocol described here are critical for conducting MU experiments safely and correctly. First, all glassware and sample handling tools that will come in contact with the reaction flask or sample need to be sterilized. Sterilization is achieved by thoroughly rinsing the items in question with ultrapure water (18.2 MΩ cm, <5 ppb TOC) and then wrapping them in aluminum foil, prior to pyrolyzing at 500°C in

air for at least 3 hours. Once the equipment has been pyrolyzed, and while preparing samples for analysis, care must be taken to avoid organic contamination. The risk of contamination can be minimized by wearing nitrile gloves, a laboratory coat, and protective eyewear. Be sure to work with samples away from one's body as common sources of contamination include finger prints, skin, hair, and exhaled breath. Avoid contact with wet gloves and do not use any latex or nylon materials. Second, thorough degassing of the reaction flask prior to gas addition into the reaction flask is critical. The presence of even small amounts of O_2 in the reaction flask pose an explosion risk when the spark is discharged into inflammable gases such as CH_4. While degassing the flask, the water inside the flask will boil, which will prevent a stable reading. At this stage there are two options: 1) degas the flask via freeze-thaw cycles (typically 3 are used), or 2) simply degas the liquid solution. In the latter case, some water will be lost, however, the amount will be relatively minor compared to the remaining volume. Third, a well-equipped and efficient setup must be carefully constructed to establish a consistent spark across the electrodes throughout the entirety of the experiment.

With regards to Tesla coil care, BD-50E Tesla coils are not designed for prolonged operation, as they are intended for vacuum leak detection. Intermittent cooling of the Tesla coil is thus recommended for extended operational lifetime. There are multiple ways of achieving this. One simple way is to attach a timer in-line between the spark tester and its power supply and program the timer such that it alternates in 1 hour on/off cycles. Cooling the Tesla coil with a commercial fan may also be necessary to prolong the life of the Tesla coil. The Tesla coil tip should be touching or almost touching one of the tungsten electrodes; a distance between the two of approximately 1

mm or less. Additionally, an intense discharge can be achieved using a length of conductive metal wire with a loop in one end draped lightly over the electrode opposite the one touching the Tesla coil to avoid breaking the seal to the contents. It is also recommended to have a second spark generator available in case the primary spark generator fails due to extended use.

There are many additional notes worth keeping in mind when carrying out various steps in the protocol outlined here. When preparing the manifold system for an experiment and using a mercury manometer, it is generally conceded that a precision of 1 mmHg is the best achievable, due to the resolution of the human eye. Some gases may present conductivity problems with resistance-based gauges. Mercury manometers present potential spill hazards, which should be prepared for in advance.

While assembling the 3 L reaction flask, the use of silicon vacuum grease can mitigate potential organic contamination, but care should be taken to remove this thoroughly between runs. Failure to do so will result in the accumulation of silica deposits during high-temperature pyrolysis, which can interfere with future vacuum seals. Additionally, the tungsten electrodes are commercially available as 2% thoriated tungsten and should be annealed into half-round ground glass fittings. Do not pyrolyze the glass-fitted tungsten electrodes in an oven. The coefficients of thermal expansion of tungsten and glass are different and heating above 100 °C may weaken the seal around the glass annealed electrodes and introduce leaks to the system. Also, ultrapure water can be introduced into the 3 L reaction flask by pouring, using care to avoid contact with any grease on the port used, or by pipetting, using a pre-pyrolyzed glass pipette. The aqueous phase in the reaction flask can be buffered, if desired. For example, Miller and

colleagues[7] buffered the solution to pH ~ 8.7 with an NH_3/NH_4Cl buffer. To do this the aqueous phase is made 0.05 M in ammonium chloride (NH_4Cl) prior to introducing it into the reaction flask. NH_4Cl of 99.5% purity, or greater, should be used. The remainder of the NH_3 is then added to the reaction flask as a gas.

In preparation for gas introduction into the 3 L reaction flask, the flask can be secured onto the manifold by placing the flask on a cork ring, set atop a lab jack and gently raising the flask assembly until a snug connection is achieved. When checking for leaks, it is worth noting that likely sources of leaks include poor seals at the junctions of the half-round ground glass joints, which attach the tungsten electrodes to the reaction flask, and the stopcock of the adapter attached to the neck of the 3 L reaction flask. If leaks from these sources are detected, carefully remove the 3 L reaction flask from the manifold, wipe these areas with clean laboratory tissue, reapply a fresh coating of vacuum grease and reattach the flask to the manifold to search for leaks. If no leaks are found, proceed to introduce gases into the reaction flask.

While introducing gases into the apparatus, gas cylinders should be securely fastened to a support. Care should be taken to introduce gases slowly. Valves on gas cylinders should be opened slowly and carefully while monitoring the manometer to avoid over-pressuring the glassware and attached fittings. It is important to note that while adding NH_3 into the reaction flask, because NH_3 is appreciably soluble in water below the pK_a of NH_4^+ (~9.2), essentially all of the NH_3 gas introduced into the manifold will dissolve in the aqueous phase, rendering the final pressure in the flask and manifold after the addition of NH_3 as the vapor pressure of water at the ambient temperature. Once this pressure is attained, one may assume the transfer is complete. The following are

examples of the calculations that must be executed in order to precisely introduce gases into the reaction flask at their desired pressures.

2.5.1 *Introduction of Gaseous NH_3*

Due to the solubility of NH_3, essentially all of it will dissolve in the aqueous phase as long as the NH_3 in the manifold is at a higher pressure than the vapor pressure of water in the reaction flask. Therefore, the ambient temperature should be noted and the vapor pressure of water at that temperature should be referenced prior to introducing NH_3 into the manifold. The target pressure of NH_3 to be introduced into the reaction flask should be equal to the target pressure of NH_3 in the 3 L reaction flask, plus the vapor pressure of water in the reaction flask, at the recorded ambient temperature. For example, at 25 °C, the vapor pressure of water is approximately 24 mmHg. Thus, in order to introduce 200 mmHg of NH_3 into the reaction flask, load roughly 225 mmHg of NH_3 into the manifold prior to transferring NH_3 from the manifold and into the reaction flask. This will result in approximately 200 mmHg of NH_3 being introduced into the reaction flask.

2.5.2 *Introduction of CH_4*

After NH_3 addition and its dissolution into the aqueous phase, the pressure in the headspace of the reaction flask will be equal to the vapor pressure of water at 25 °C, approximately 24 mmHg. This value will be used, in conjunction with the example manifold shown in Figure 2.2, to carry out a calculation for how much CH_4 to introduce into the manifold such that 200 mmHg of CH_4 will be introduced into the reaction flask:

P_1 = total pressure desired throughout the entire system, including the reaction flask
V_1 = total volume of the entire system, including the reaction flask
P_2 = pressure of CH_4 needed to fill manifold prior to introduction into reaction flask
V_2 = volume of manifold used for gas introduction
P_3 = pressure already in the headspace of the reaction flask
V_3 = volume of the reaction flask

P_1 = 200 mmHg of CH_4 + 24 mmHg of H_2O = 224 mmHg
V_1 = (3000 + 100 + 300 + 40 + 20 + 3000 + 40 + 500) mL = 7000 mL
P_2 = pressure of CH_4 being calculated for
V_2 = (100 + 300 + 40 + 20 + 3000 + 40 + 500) mL = 4000 mL
P_3 = 24 mmHg of H_2O
V_3 = 3000 mL

$$P_1V_1 = P_2V_2 + P_3V_3 \rightarrow P_2 = \frac{P_1V_1 - P_3V_3}{V_2} \rightarrow$$

$$P_2 = \frac{(224\ mmHg)(7000\ mL) - (24\ mmHg)(3000\ mL)}{4000\ mL} \rightarrow$$

$$P_2 = 374\ mmHg\ CH_4$$

2.5.3 Introduction of N_2

After introduction of CH_4, the headspace of the reaction flask is occupied by 200 mmHg of CH_4 and 24 mmHg of H_2O for a total of 224 mmHg. This value will be used, along with the dimensions of the example manifold shown in Figure 2.2, to calculate the N_2 pressure that needs to be introduced into the manifold such that 100 mmHg of N_2 will be introduced into the reaction flask:

P_1 = total pressure desired throughout the entire system, including the reaction flask
V_1 = total volume of the entire system, including the reaction flask
P_2 = pressure of N_2 needed to fill manifold prior to introduction into reaction flask
V_2 = volume of manifold used for gas introduction
P_3 = pressure already in the headspace of the reaction flask
V_3 = volume of the reaction flask

P_1 = 24 mmHg of H_2O + 200 mmHg of CH_4 + 100 mmHg of N_2 = 324 mmHg
V_1 = (3000 + 100 + 300 + 40 + 20 + 3000 + 40 + 500) mL = 7000 mL
P_2 = pressure of N_2 being calculated for
V_2 = (100 + 300 + 40 + 20 + 3000 + 40 + 500) mL = 4000 mL
P_3 = 200 mmHg of CH_4 + 24 mmHg of H_2O = 224 mmHg
V_3 = 3000 mL

$$P_1V_1 = P_2V_2 + P_3V_3 \rightarrow P_2 = \frac{P_1V_1 - P_3V_3}{V_2} \rightarrow$$

$$P_2 = \frac{(324\ mmHg)(7000\ mL) - (224\ mmHg)(3000\ mL)}{4000\ mL} \rightarrow$$

$$P_2 = 399\ mmHg\ N_2$$

After initiating the experiment, the system must be checked regularly to ensure the experiment is running properly. Things to check include: 1) the spark generator is producing a spark, and 2) the spark is being generated across in a continuous manner. If the above conditions are not met, disconnect the Tesla coil from its power supply and replace it with the backup Tesla coil. Meanwhile, repairs to the malfunctioning Tesla coil can be made. Often times, the contact plates inside the spark generator housing can become corroded from extended use and should be polished, or replaced.

Upon completion of the experiment, the gases in the head-space may be irritating to the respiratory system. Harmful gases, such as HCN[3] can be produced by the experiment. If the experimenter is not collecting gas samples for analysis, it may be helpful to connect the apparatus to a water aspirator to evacuate volatiles for approximately one hour after completion of the experiment, while the apparatus remains in the fume hood, prior to collecting liquid samples. For safety reasons, it is advised that the apparatus is vented in a fully-operational fume hood. Sample collection should be performed in an operational fume hood and sample handling in a positive-pressure, high efficiency particulate air filtered flow bench is recommended.

Among the numerous types of products formed by spark discharge experiments, AAs are of significance. AAs are synthesized readily via the Strecker synthesis.[15] This is, of course, but one mechanism of synthesis, and others may also be operative, such as

direct amination of precursors including acrylonitrile to give β-alanine precursors, or direct hydrolysis of higher molecular weight tholin-like material to give AAs directly, by-passing the Strecker mechanism.

AA contamination of the samples produced by MU experiments can occur if the precautions mentioned earlier are not followed explicitly. During sample analysis, it is important to search for signs of terrestrial contamination that may have originated from sample handling or sample storage. The use of OPA/NAC[14] in conjunction with LC-FD techniques allows for the chromatographic separation of D- and L-enantiomers of AAs with chiral centers and their respective, individual quantitation. Chiral AAs synthesized by the experiment should be racemic. Acceptable experimental error during the synthesis of AAs with chiral centers is generally considered to be approximately 10%. Therefore chiral AA D/L ratios suggestive of enrichment in one enantiomer by more than 10% is a good metric by which to determine if the sample has been contaminated.

The methods presented here are intended to instruct how to conduct a MU type spark discharge experiment; however, there are limitations to the technique described here that should be noted. First, heating the single 3 L reaction flask (Figure 2.1.B), will result in condensation of water vapor onto the tips of the electrodes, dampening the spark, and reducing the generation of radical species that drive much of the chemistry taking place within the experiment. Furthermore, the use of a heating mantle to heat the apparatus is not necessary to synthesize organic compounds, such as AAs. This differs from Miller's original experiment where he used a more complex, custom-built, dual flask apparatus and heated the small flask at the bottom of the apparatus, which had water in it (Figure 2.1.A).[2] Heating the apparatus helped with circulation of the starting

materials and aimed to mimic evaporation in an early Earth system. Second, the protocol detailed here recommends a 1 hour on/off cycle when using the Tesla coil, which effectively doubles the amount of time an experiment takes to complete, compared to the experiments conducted by Miller, as he continuously discharged electricity into the system.[3] Third, as spark generators are not intended for long-term use, they are prone to malfunction during prolonged use and must be regularly maintained and sometimes replaced by a back-up unit, if the primary spark generator fails during the course of an experiment. Last, this protocol involves the use of glass stopcocks, which require high vacuum grease to make appropriate seals. If desired, polytetrafluoroethylene (PTFE) stopcocks can be used to avoid vacuum grease. However, if examining these stopcocks for potential leaks with a spark leak detector, be cautious to not overexpose the PTFE to the spark as the spark can compromise the integrity of the PTFE and lead to poor seals being made by these stopcocks.

The significance of the method reported here with respect to existing techniques, lies within its simplicity. It uses a commercially available 3 L flask, which is considerably less fragile and easier to clean between experiments than the original design used by Miller.[3] Because the apparatus is less cumbersome, it is small enough to carry out an experiment inside a fume hood.

Upon mastering this protocol, it can be modified in a variety of ways to simulate numerous primitive terrestrial environments. For example, more oxidized gas mixtures can be used.[12, 16, 17] Furthermore, using modifications of the apparatus, the energy source can be changed, for example, by using a silent discharge,[3] ultraviolet light,[18] simulating

volcanism,[3, 10, 19] imitating radioactivity from Earth's crust,[20] and mimicking energy produced by shockwaves from meteoritic impacts,[21] and also cosmic radiation.[16, 17]

The classic MU experiment demonstrated that AAs, important building blocks of biological proteins, can be synthesized using simple starting materials under simulated prebiotic terrestrial conditions. The excitation of gaseous molecules by ED leads to the production of organic compounds, including AAs, under such conditions. While AAs are important for contemporary biology, the MU experiment only provides one possible mechanism for their abiotic synthesis, and does not explain the origin of life, as the processes that give rise to living organisms were likely more complex than the formation of simple organic molecules.

2.6 List of Materials Used

Table 2.2 Materials, and associated part numbers, used for conducting ED experiments.

Name	Company	Catalog Number	Comments
Glass Plugs for Manifold	Chemglass	CG-983-01	
High Vacuum Grease	Apiezon	N/A	Type M/N
Silicon High Vacuum Grease	Dow Corning	1597418	
Teflon PFA Tubing	McMaster-Carr	51805K54	
Ultra-Torr Vacuum Fittings	Swagelok	SS-4-UT-6	
Dry Scroll Vacuum Pump	Edwards	A72401905	
U-Tube Manometer	Alta-Robbins	100SS	
Tungsten Electrodes	Diamond Ground Products	TH2-1/16	2% thoriated
Methanol	Alfa Aesar	N/A	Ultrapure HPLC Grade
Teflon-Coated Magnetic Stir Bar	McMaster-Carr	5678K127	
Gaseous NH3	Airgas	AMAHLB	99.99% purity
Gaseous CH4	Airgas	ME UHP300	99.99% purity
Gaseous N2	Airgas	NI UHP300	99.999% purity
Tesla Coil	Electro-Technic Products	15001	Model BD-50E
24 hr Plug-in Basic Timer	General Electric Company	15119	
Cleaning Detergent	Alconox	1104	
Toluene	Thermo Fisher Scientific	N/A	Optima Grade
Luna Phenyl-Hexyl HPLC Column	Phenomenex	00G-4257-E0	Brand: Luna
Formic Acid	Sigma-Aldrich	F0507	Used for 50 mM NH_4Fo

2.7 Acknowledgements

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CHAPTER 3: AMINO ACIDS GENERATED FROM HYDRATED TITAN THOLINS: COMPARISON WITH MILLER-UREY ELECTRIC DISCHARGE PRODUCTS

Adapted with permission from:
Cleaves, H. J. II; Neish, C.; Callahan, M. P.; Parker, E.; Fernández, F. M.; Dworkin, J. P. (2014) Amino acids generated from hydrated Titan tholins: Comparison with Miller-Urey electric discharge products. *Icarus*, 237, 182-189.

3.1 Abstract

The protocol outlined in the previous chapter helps better understand the synthesis of molecules important for life under possible early Earth conditions, but can also be useful for understanding extraterrestrial organic compound synthesis, such as on Titan, the largest moon of Saturn. Titan chemistry has been an area of intense research for its potential to aid the future search for life. To evaluate the ability of MU ED reactions to mimic extraterrestrial environments, a comparison study was conducted between MU ED experiments and Titan analog experiments. Here, various analogs of Titan haze particles (termed 'tholins') have been made in the laboratory. In certain geologic environments on Titan, these haze particles may come into contact with aqueous NH_3 solutions, hydrolyzing them into molecules of astrobiological interest. A Titan tholin analog hydrolyzed in aqueous NH_3 at room temperature for 2.5 years was analyzed for AAs using highly sensitive UPLC-FD/TOF-MS after derivatization with a fluorescent tag. We compare here, AAs produced from this reaction sequence with those from room temperature MU ED reactions. Most of the AAs detected in low temperature MU $CH_4/N_2/H_2O$ ED reactions are generated in Titan simulation reactions. This suggests that many processes provide very similar mixtures of AAs. Although it is unknown how life

began, it is likely that given reducing conditions, similar materials were available throughout the early Solar System to facilitate chemical evolution.

3.2 Introduction

It is generally believed that life on Earth originated from environmentally supplied organic precursors.[1-4] A variety of possible sources have been involved for such an "Oparin-Haldane" type origin of life, including atmospheric synthesis,[5-7] extraterrestrial delivery from meteorites and comets,[8] and geothermal synthesis.[9, 10]

Although the composition of the atmosphere at the time of the origin of life is debated,[11-13] other Solar System bodies have atmospheres that may be conducive to organic synthesis. One example is Saturn's moon Titan, which has an upper atmosphere consisting of ~98% N_2 and 2% CH_4, along with minor amounts of other species including H_2, HCN, CO, and organics such as ethane, ethylene, acetylene, and cyanoacetylene.[14] However, due to Titan's low temperature (~95 K), most of its water is present as ice in the crust and mantle, acting similar to bedrock on Earth. In contrast, the early Earth was likely above the freezing point of water for long periods of time, with possible early excursions above and below the conditions at which water can exist as a liquid.[15, 16]

Titan's atmosphere is exposed to various types of energetic processing, producing higher molecular weight organics. When produced under laboratory conditions, these polymers are collectively known as tholins.[17] Similar materials have also been suggested to be formed in the atmospheres of Triton and Pluto.[18] On Titan, it has been proposed that as the organic haze particles precipitate from the atmosphere they may come in contact with eutectic brines highly enriched in NH_3, in short-lived 'oases' of liquid water formed

through impact melting or cryovolcanism.[19] These interactions could lead to reactions producing molecules of biological interest, for example AAs and nucleobases.[20-22]

The energetic processing of Titan's present atmosphere is likely somewhat different than that which acted upon the primitive Earth's,[8] or that which contributed to interstellar ice grain organic formation.[23, 24] For example, Earth has always received a much higher flux of solar radiation than Titan, and the flux in interstellar environments is attenuated distinctly due to the differences in path lengths, composition, and density of the medium. However, it was noted almost 30 years ago that ED reaction products and organic molecules found in carbonaceous chondrites are very similar with respect to their AA contents.[25] The inference is that there is some similarity in the mechanism of formation in both cases,[26] for example *via* Strecker-like synthesis from small reactive organic precursors, such as ketones or aldehydes.[27] Analytical studies since the 1970s have revealed a much richer molecular complexity in many carbonaceous chondrites.[28, 29] It is worth bearing in mind the various degrees of post-accretional processing the natural samples have undergone over ~4.5 Ga compared to the laboratory samples, and that, with few exceptions,[30-31] laboratory samples have not received the same degree of scrutiny.

A number of laboratories have modeled the synthesis of Titan haze particles using CH_4/N_2 gas mixtures at low temperature using various energy sources, such as ED and UV light[18, 20, 32-37]. A subset of these laboratories also investigated the products of tholin hydrolysis, which is the subject of this chapter.

There are numerous variables involved in these simulations, which could render comparison difficult, for example, the conditions under which the tholins are synthesized,

and their products processed and analyzed (Figure 3.1); nevertheless, as we will show, the detected products remain remarkably similar despite these variables.

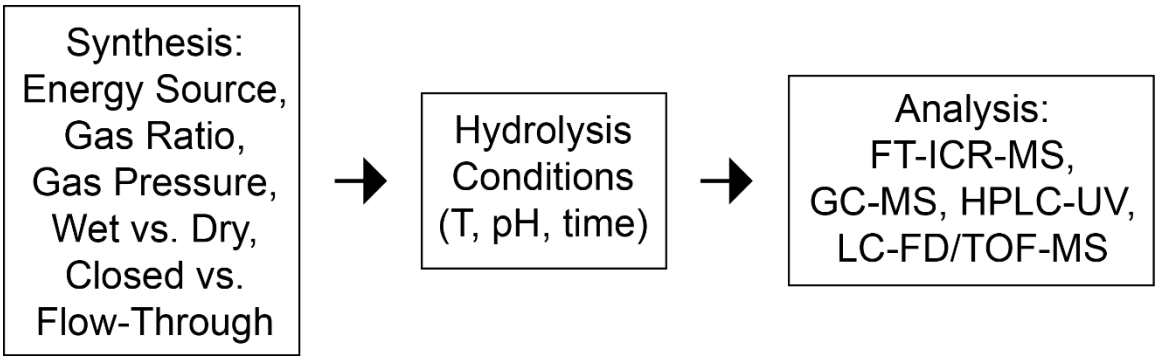

Figure 3.1. Some variables involved in the preparation of AA yielding tholins reported in the literature. FT-ICR-MS = Fourier transform-ion cyclotron resonance-mass spectrometry.

For example, during tholin synthesis, a wide-range of ratios of N_2 to CH_4 (from ~1000:1 to 1:1), total gas pressures (from ~400 mmHg to 1 mmHg) and reaction temperatures (from 296 K to 195 K) have been used. Some use flow reactors, while others use static ones. Workup has ranged from high temperature (>100 °C) aqueous acid to extremely low temperature (-20 °C) aqueous base over widely varying time ranges. Lastly, a variety of techniques have been used to measure AA products of these reactions.

A short historical note regarding the terminology used here is useful for clarifying this discussion. While there was some experimental work on the generation of discrete low molecular weight organic compounds, such as AAs, using EDs acting on various gas mixtures prior to Miller's pioneering 1953 publication[5] (see for example, Löb[38]), no formal name was applied by Miller or his predecessors to the complex organic mixtures that result from such experiments. It should be stressed that Miller's experiments were produced in the context of a reducing early Earth atmosphere, which adds a level of

intentional contextualization to the materials produced. Furthermore, Miller recognized that discrete organic compounds were isolated from hydrolysis of the products of the ED reaction, which could include a mixture of those synthesized directly in the discharge, those made in aqueous phase reactions from intermediates produced in the gas phase, and those derived from hydrolysis of larger, more complex organic polymers produced in either phase. Collectively these were termed "ED products".[A]

Thus the principle practical distinctions between MU ED products and tholins, are that MU products can be considered as alternatively: (1) organic materials derived from the action of EDs acting on reduced gases, (2) organic materials derived from energetic (be it *via* ED, UV, or high energy particle) processing of gas mixtures (reducing or otherwise) simulating planetary atmospheric chemistry, or (3) chemistry as described in (2) in the presence of a liquid phase. Tholins, in contrast, are generally considered to be the resulting non-volatile organic products derived from reduced gases *via* any type of energetic processing simulating Solar System environments at temperatures below the freezing point of water. In both cases, the materials are considered to be precursors, which require some workup for the release of biomolecules. In neither case is the potential importance of the molecular weight of the precursors considered significant, *i.e.*

[A] It was not until Sagan and co-workers recognized the potential parallels between MU reactions and the chemistry of the atmospheres of the Jovian planets and their satellites that the term "tholin" was coined, which Sagan derived from the Greek root "tholos" ("θολοσ"). It appears Sagan intentionally chose a Greek root, which could be alternatively interpreted as being derived from the word meaning "muddy" or "unclear", referencing both the color and molecular complexity and heterogeneity of these materials, as well as from the root meaning "dome", referencing their astronomical significance. In a personal communication from Miller to Sagan discussing Sagan's suggested name, Miller, tongue-in-cheek, suggested "tholin" was a poor choice of terminology, partly because the English voiceless dental fricative "Θ" ("th-") would be difficult to pronounce in German (where it would be pronounced as "/d/") (Miller, personal communication to HJC).

either or both might liberate small molecules in water, such as HCN, NH_3, and aldehydes and ketones. These small molecules may undergo secondary reactions, such as the Strecker reaction, to give the final observed products, including AAs.

The salient points we wish to make are that there is considerable similarity between tholins and MU discharge products when derived from CH_4 and N_2, with respect to the AAs, and potentially other products, produced regardless of the temperature of reaction, or the presence of liquid water during energetic processing. This suggests that the gas phase chemistry of non-oxygen-containing species dominate the AA synthesis pathways, and that as these are highly transient species, the temperature may be of little importance.[39] The point may have considerable consequence for the types of organic materials, which can be expected to be delivered to almost any reducing planetary environment throughout the universe.

Among the various other studies of these materials,[31, 40] it has often been of interest to measure AAs produced from these simulations. As mentioned above, the paucity of water, or other O-containing species in Titan's atmosphere, means that the carboxyl and side chain oxygen must be derived from a step simulating some sort of aqueous surface chemistry, or *via* a laboratory hydrolysis step.

Recent advances in mass spectrometry have allowed unprecedented high resolution accurate mass analysis of complex prebiotic mixtures, including FT-ICR-MS studies of the Murchison meteorite and Titan tholins.[28, 40, 41] AAs have been detected in several studies modeling these types of processes, though the techniques used have limited sensitivities for confident identification and quantification. For example, Khare et al.[39] detected 16 AAs using GC-MS, while McDonald et al.[18] found a partially

overlapping set of 15 AAs using HPLC with UV detection. More recently, Neish et al.[22] found species with the exact masses of 18 of the coded AAs not containing sulfur using FT-ICR-MS, but only four of these were confirmed as the correct structural isomer using tandem mass spectrometry. Hörst et al.[42] detected 14 of the non-sulfur coded AAs, though only alanine and glycine, could be confirmed using GC-MS.

We report here, for the first time, on the application of high sensitivity UPLC-FD/TOF-MS for the analysis of concentrated aqueous NH_3-processed tholins and their comparison with the products of a MU $CH_4/N_2/H_2O$ ED experiment. We find a large number of AAs and amine products. The distribution of AAs between samples is very similar, though there are some differences, which speak to nuances in these molecules' modes of formation. The importance of the differences and similarities is discussed here.

3.3 Materials and Methods

3.3.1 *Chemicals and Reagents*

All reagents used in the analyses reported here were purchased from Fisher Scientific or Sigma-Aldrich, and were of reagent grade or higher unless otherwise stated. All tools used to handle the samples were cleaned using Millipore Direct Q10 H_2O (18.2 MΩ cm, <3 ppb total organic carbon, hereafter mQH_2O) before wrapping them in aluminum foil and heating them in air overnight at 500 °C.

3.3.2 *Titan Tholins*

Titan tholins were generated as described in Neish et al.[21] by subjecting a mixture of 2% CH_4 and 98% N_2 to an AC ED at a pressure of 880 Pa and 195 K in a glass reaction vessel. 50 mg of tholin were dissolved in 2 mL of acetonitrile, and 100 µL of this solution was further diluted into 2 mL of a 1:1 mixture of 14 N aqueous NH_4OH (EMD

Chemicals Inc.) and doubly deionized water. This solution was allowed to sit at room temperature for 2.5 years. As this sample did not contain significant amounts of inorganic salts it was derivatized and analyzed directly after hydrolysis (*i.e.* without desalting *via* IEC). However, we found that it was helpful to include a room temperature vacuum drying step prior to derivatization, to remove excess NH_3, which often led to a large derivatized NH_3 peak that interfered with analysis.

3.3.3 *MU ED Polymers*

MU ED polymers were prepared using the general protocol outlined by Parker et al.,[43] while implementing the starting conditions specified in Ring et al.,[44] except the NH_3 buffer was replaced with 0.225 M $NaHCO_3$ adjusted to pH 8.7, which was degassed prior to introduction of the gas mixture. These were analyzed, after desalting, by IEC as described in Johnson et al.,[30] both directly and after vapor phase acid hydrolysis. Table 3.1 shows a comparison of previously reported results using different methods of synthesis and processing.

3.3.4 *Standard Analysis*

Stock AA solutions (~10^{-3} M) were produced by mixing individual AAs (97-99% purity) in mQH_2O. Three reagents were prepared for derivatization and analysis by a Waters ACQUITY UPLC and FD and a Waters LCT Premier TOF-MS, as per Glavin et al.[45]: (1) 0.4 M sodium borate (pH 9.4), (2) 0.1 M hydrazine hydrate, and (3) OPA/NAC. The 18 non-sulfur-containing coded AAs were used as standards, as well as D,L-α-hydroxymethylalanine, α-hydroxymethylserine, β-hydroxyaspartic acid, D,L-ornithine, D,L-α-amino adipic acid, D,L-homoserine, D,L-2-methylglutamic acid and D,L-Ise.

Table 3.1. Comparison of the various experimental conditions and analytical techniques that have been used to study MU reaction products and Titan tholins.

Study	MU – NH_3(aq)[a,b]	MU – no NH_3[c]	Titan Tholin[d]	Titan Tholin[e]	Triton Tholin[f]	Titan Tholin[g]
Gas Mixture (pressure, mmHg)	N_2 (200), CH_4 (200), H_2O (14)	N_2 (200), CH_4 (200), H_2O (14)	N_2 (6.3), CH_4 (0.13)	N_2 (0.135), CH_4 (0.015)	N_2 (0.999), CH_4 (0.001)	N_2 (0.735), CH_4 (0.015)
Solution Phase[h]	0.0675 M NH_4Cl, pH 8.7[i]	0.225 M $NaHCO_3$, pH 8.7[i]	None	None	None	None
Energy Source	ED	ED	ED	ED	ED	ED
Temperature	298 K	298 K	Gas phase: 195 K	298 K	298 K	170 K
Workup	3 N HCl hydrolysis, 373 K, 24 hr	Straight/6 N HCl vapor hydrolysis, 373 K, 24 hr	15 wt% aq. NH_3, pH ~11.5, 253 K	6 N HCl, 373 K, 20 hr, IEC	HCl/ propionic acid 150 °C, 85 min	25 wt% aq. NH_3, 253 K
Derivatization	None	OPA/NAC	OPA/NAC	None	Waters Picotag	None
Analysis	GC-MS	LC-FD/TOF-MS	FT-ICR-MS, LC-FD/ TOF-MS	GC-MS	HPLC-UV	GC-MS

[a,b] As reported in Ring et al.[44] and Wolman et al.[25]
[c] This paper.
[d] This paper, also reported in Neish et al.[22]
[e] As reported in Khare et al.[39]
[f] As reported in McDonald et al.[18]
[g] As reported in Poch et al.[46]
[h] If present, exposed to gas during energetic gas processing.
[i] Initial pH

The NH_4Fo buffer used for UPLC-FD/TOF-MS analyses was made by titrating 50 mM formic acid to pH 8 with NH_4OH and adding methanol to a final concentration of 8 vol.%.

3.3.5 *UPLC-FD/TOF-MS Analysis*

Sample extracts were analyzed by UPLC-FD/TOF-MS, as described in Johnson et al.[30] Sample extracts were prepared as described above and derivatized OPA/NAC with the following modification. The samples were derivatized for 1 min, after which, the reaction was quenched with 75 µL of hydrazine. Samples were then immediately placed into the UPLC-FD/TOF-MS autosampler and injected. Details of the TOF-MS settings and the AA quantification methods used for these analyses are detailed elsewhere.[45] The UPLC-FD/TOF-MS technique had a limit of detection (LOD) in the low femtomole range for AAs.

Products were identified based on three criteria: (1) the fact that they must be amine-functional group-containing molecules to produce fluorescence signals, (2) the correspondence of their chromatographic retention time with that of a known standard, and (3) their protonated molecular (parent) ions in the mass spectrum.

3.4 Results and Discussion

Examination of the chromatograms obtained reveals a complex suite of fluorescent products (Figure 3.2). These should contain primary amino groups as to be fluorescent given the excitation and emission wavelengths should require the presence of an OPA tag. However, in the case of samples that were not subjected to IEC, there may also be other untagged compounds, which are inherently fluorescent under these conditions, present in these chromatograms.

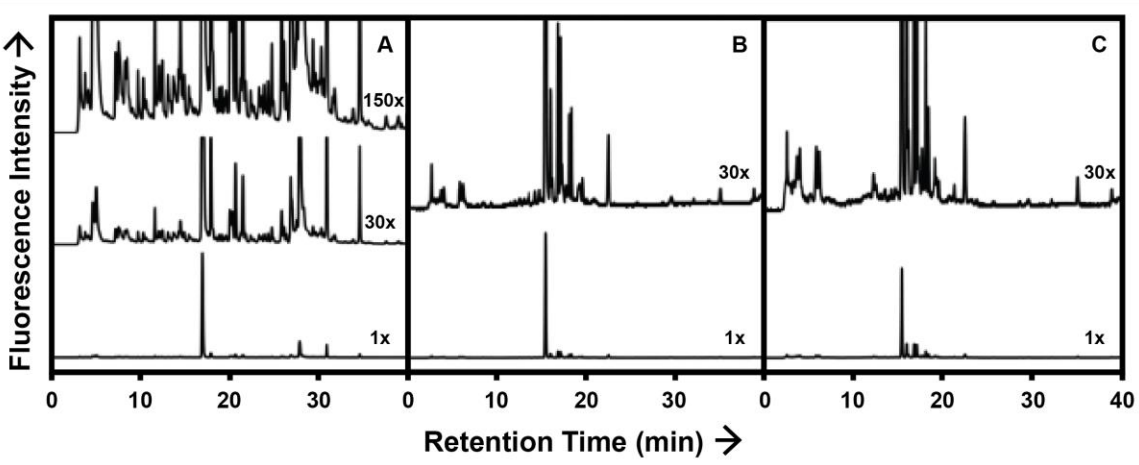

Figure 3.2. UPLC-FD traces showing the abundances of likely OPA/NAC tagged peaks of (A) the low temperature ammonialyzed tholin; (B) the HCl vapor hydrolyzed carbonate-buffered spark product; and (C) the H_2O hydrolyzed carbonate-buffered spark product, showing magnifications. B and C are to scale.

Closer examination of this data reveals even greater amine diversity (Figure 3.2.A, middle trace, Figure 3.2.B/C, top traces). Zooming in further on the baseline, an even larger diversity of amines is noticed (Figure 3.2.A, top trace). The diversity is almost fractal, and its visualization may be limited by chromatographic peak capacity. The baseline complexity of the Titan tholin chromatogram is not completely mirrored in the MU samples, however, some of this may be due to volatile components lost during the IEC and workup of the MU samples, such as small primary amines, and others may be inherently fluorescent molecules which are not retained by IEC.[47]

Examination of the extracted ion chromatograms (EICs) allows more confident identification of these AAs, including their enantiomers (Figure 3.3).

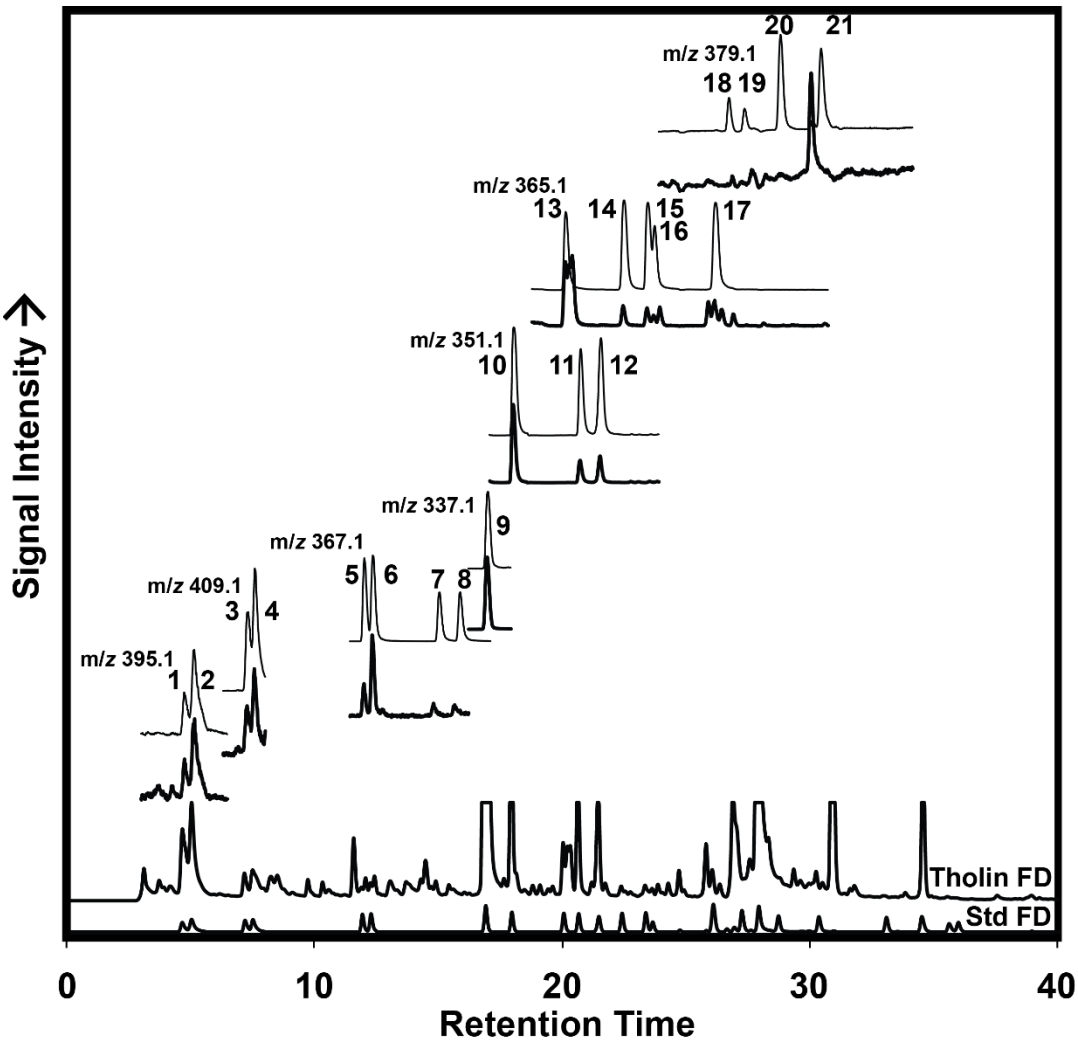

Figure 3.3. Fluorescence traces and EICs of various AAs searched for in the Titan tholin products and an authentic standard. (1 and 2) D,L-aspartic acid, (3 and 4) D,L-glutamic acid, (5 and 6) D,L-serine, (7 and 8) D,L-isoserine, (9) glycine, (10) β-alanine, (11 and 12) D,L-alanine, (13) γ-aminobutyric acid, (14) D-β-aminobutyric acid, (15) L-β-aminobutyric acid, (16) α-aminoisobutyric acid, (17) D,L-α-aminobutyric acid, (18 and 19) D,L-isovaline, (20 and 21) D,L-valine. Bottom: fluorescence trace, AA standard, above that, fluorescence trace of the Titan tholin analogue. Shown floating above are the EICs of the OPA/NAC traces, with the standard traces above the tholin traces. All EICs are traces of the protonated molecular ion of the given OPA/NAC labeled AAs with a ±0.1 amu mass window.

Among the coded AAs detected in the tholin sample were glycine (Gly), D,L-alanine (ala), D,L-serine (Ser), D,L-aspartic acid (Asp), D,L-glutamic acid (Glu), D,L-asparagine (Asn), and D,L-glutamine (Gln). Among the non-coded AAs detected in the

tholin sample were D,L-isoserine (Ise), β-alanine (β-Ala), γ-aminobutyric acid (γ-ABA), D,L-β-aminobutyric acid (β-ABA), α-aminoisobutyric acid (α-AIB), D,L-α-aminobutyric acid (α-ABA) and D,L-isovaline (Iva). Among the coded AAs not detected within the detection limit of this analysis were valine (Val), leucine (Leu), isoleucine (Ile), histidine (His), arginine (Arg), phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp) and lysine (Lys) (Figure 3.4). These results show some similarities to those of Neish et al.,[22] who detected Asn, Asp, Glu, and Gln in the sample, but lacked the capability to detect species as small as Gly, Ala, and Ser.

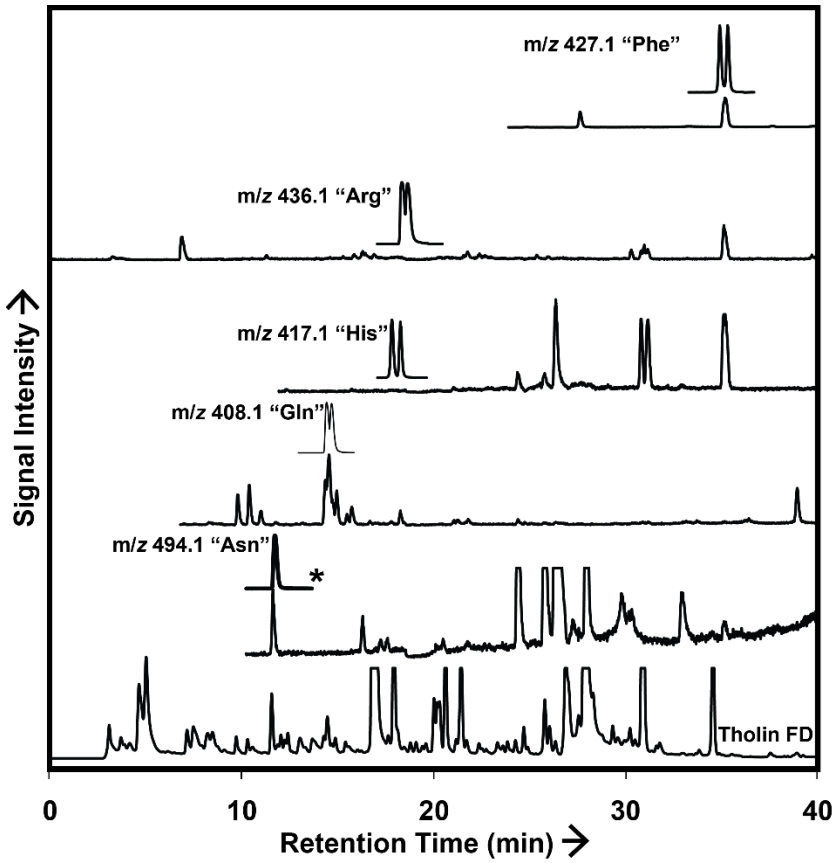

Figure 3.4. Fluorescence chromatogram and EICs of Gln, Asn, and other coded AAs searched for, along with EICs from authentic standards. Order of EICs is as stated for Figure 2. His, Arg, and Phe were not detected, nor were D,L-Trp or D,L-Tyr (data not shown).

Gln, Asn and Trp are not typically detected after high temperature (100 °C) acid hydrolysis, due to their hydrolytic instability,[48] which explains their scarcity in the acid hydrolyzed MU samples. However Asn and Gln survive the low-temperature hydrolysis conditions to which the Titan tholin was exposed, and the hot water hydrolysis conditions in the case of the MU sample. The failure to detect the others could be due to their being present in quantities below the detection limit of our analysis, or their absence. This work emphasizes the importance of chromatographic analyses beyond the simple infusion and identification of the exact mass of a species through FT-ICR-MS, since isobaric structural isomers may be present. For example, in the case of His, we observe what appears to be an enantiomeric pair. Such a scenario suggests an additional species shares the molecular formula of His, one stereocenter, and a single primary amino group (or it would derivatize twice, giving it a different parent mass). Yet, it likely also contains one free carboxyl group, otherwise it would not elute on the time-scale observed. Nonetheless, it should be noted that many other primary amine-containing structural isomers are possible. This agrees with the results of,[22] who found evidence for a structural isomer of His with a different MS/MS fragmentation pattern than the pure substance.

Also identified in all reactions were significant amounts of isoserine and various low molecular weight amines, including methylamine, ethylamine, and ethanolamine. Since some of these are volatile and may have been lost during the evaporation steps of sample workup, they could not be meaningfully quantified and compared here. Comparing data from previous measurements of Tholin AAs,[22, 39] ED reactions,[25, 44] and our current data is presented in Figure 3.5. We have focused on a subset of the AAs generated, which are among the most abundant. This subset is amenable to primary

amine analysis using OPA/NAC. This technique does not allow for the analysis of secondary amines, such as sarcosine and proline, despite sometimes being significant components of such samples. For example, sarcosine is produced in ~13% and proline in 0.3% of the relative molar abundances of glycine in some low temperature MU experiments.[44] Comparison of peak areas with those of known standards allows quantification, which is shown in Table 3.2.

For the comparison below, the yields of AAs from various tholin syntheses are binned by the number of carbon atoms per molecule (C_2: Gly, C_3: D,L-Ser, D,L-Ise, D,L Ala, β-Ala; C_4: D,L-Asp, γ-ABA, α-AIB, D,L-β-ABA; D,L-α-ABA; C_5: D,L-Glu).

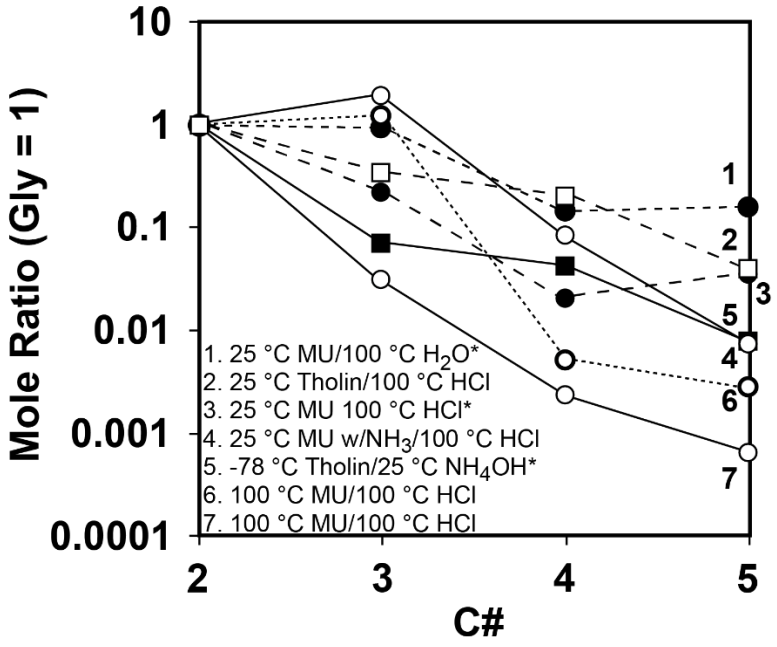

Figure 3.5. Molar ratios of AAs as a function of carbon number for various CH_4/N_2 experiments. Closed symbols, this paper; open symbols, literature data; circles, MU; squares, Titan. The inset provides reference to experimental details (reaction temperature/hydrolysis conditions). Asterisks indicate data from present analysis. (1) 298 K MU experiment; (2) 298 K Dry Titan tholin[39]; (3) 298 K MU experiment; (4) 298 K MU experiment using single chambered apparatus[44]; (5) Cold Titan tholin[22] (data this study); (6) Hot MU using double chambered volcanic apparatus[30]; (7) Hot MU using double chambered silent discharge apparatus[30].

Table 3.2. Yields of AAs from aqueously ammonialyzed Titan Tholin analog and water and acid hydrolyzed ED polymer.

Sample Compound	Titan Tholin[a]		$NaHCO_3$ buffered MU[b]		$NaHCO_3$ buffered MU[c]	
	Molar Ratio[d]	D/L[e]	Molar Ratio[d]	D/L	Molar Ratio[d]	D/L
Gly[f]	1	-	1	-	1	-
D,L-Asp	7×10^{-2}	1.00	7×10^{-2}	0.77	3×10^{-2}	0.60
D,L-Ala	6×10^{-2}	1.05	0.3	1.07	9×10^{-2}	1.01
β-Ala	4×10^{-2}	-	0.2	-	3×10^{-2}	-
D,L-Asn	2×10^{-2}	nd	1×10^{-2}	nd	5×10^{-4}	nd
D,L-Glu	2×10^{-2}	1.24	0.1	0.73	4×10^{-2}	1.02
γ-ABA[f]	1×10^{-2}	-	6×10^{-5}	-	2×10^{-3}	-
D,L-Gln	1×10^{-2}	0.85	0.2	1.03	0	nd
D,L-Ise	1×10^{-2}	1.15	1×10^{-2}	1.02	2×10^{-2}	0.78
D,L-Ser	9×10^{-3}	0.75	3×10^{-2}	1.27	9×10^{-3}	0.78
D,L-α-ABA	8×10^{-3}	nd	6×10^{-2}	nd	3×10^{-2}	nd
α-AIB[f]	6×10^{-3}	-	3×10^{-2}	-	1×10^{-2}	-
D,L-β-ABA	6×10^{-3}	1.00	1×10^{-2}	1.29	3×10^{-3}	1.16

[a] The recovery of glycine was 9.1 mg per g of polymer.

[b] H_2O hydrolyzed. The recovery of glycine was 1.13 mmoles per mole of input CH_4.

[c] HCl hydrolyzed. The recovery of glycine was 1.44 mmoles per mole of input CH_4. Note the recovery of glycine from the HCl hydrolyzed MU experiment was 1.27x that of the H_2O hydrolyzed sample. The relatively greater recovery of glycine may account for some of the apparent reduction in relative yields of other AAs for the HCl hydrolyzed MU experiment.

[d] Relative to Gly = 1.

[e] Ratio of the detected D and L enantiomers, where measureable. An en dash indicates there are no stereoisomers for the compound. nd = not determined, as the enantiomers were not chromatographically resolved.

[f] Indicates the compound in question is achiral.

The most notable differences are in the ratios of C_2:C_3 AAs, with the major contributor being alanine. Relative yields are similar in many ways, as evidenced by several studies. Modifications of the original MU apparatus set up, for example those including H_2S or nebulized water vapor, produced AA mixtures that are even more similar than that used for Peltzer et al.'s[26] comparison.[30, 49, 50] While these variations have not been systematically explored, we suggest here that multiple mechanisms could explain the similarity of one to another. These molecules are very reminiscent of the molecules produced from MU type experiments, suggesting there are similarities in their modes of formation. The laboratory experiments of Khare et al.[39] and McDonald et al.[18] were carried out at room temperature, however, these authors argued that because of the large difference in effective temperature between the energy source for molecular dissociation and the ambient temperature, both in the laboratory and on Titan, the choice of reaction temperature may not significantly affect the results.

It can be difficult to compare the results of studies using different analytical techniques. Some species abundant in these samples may not be readily detected by our, or other, techniques, possibly making various samples appear similar. For example, proline, sarcosine, and 1,2-diamines are not detectable using our techniques. Raulin and colleagues detected three AAs (Gly, Asp and Ala) after N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide derivatization of similar materials, using GC-MS analysis.[46] These species are also detectable using the present methodology, which is more sensitive. Gly was easily detected as were a large suite of other AAs, including Ala, Asn, Gln, Asp, Glu, Ser, and Ise. Additionally, our method allows enantiomeric separation, thus we can determine the potential degree of terrestrial contamination.

The MU ED samples were made from CH_4 and N_2 at room temperature in the presence of water vapor buffered with aqueous $NaHCO_3$, while the Titan tholin samples were made from a high frequency discharge at low temperature in the absence of water, and then hydrated in aqueous NH_3. ED experiments starting from N_2 or NH_3, CH_4, and H_2O likely generate such radical species as N and CH_3, which recombine to yield HCN, acetylene and cyanoacetylene.[51, 52] The deposition of these species into aqueous solutions containing NH_3 allows for Strecker synthesis of AAs, and other reactions to yield AAs.[27]

Strecker- and Michael-addition-type reactions proceed directly in the aqueous phase starting with the organic precursors, such as HCN, aldehydes, acrylonitrile and cyanaoacetylene. Aqueous HCN chemistry, itself, may produce some higher carbon skeletons, such as those of Asp and Glu.[53] However, the mechanism for the generation of Glu from HCN is not yet well understood.[54]

If these compounds are derived from non-oxygenated species, mechanistic pathways for their synthesis must exist, which most likely are distinct from classically proposed ones, such as the Strecker synthesis, as aldehydes and ketones cannot be initial precursors for their formation. This does not mean that processes involving aldehydes or ketones cannot also contribute to these processes, but that both types of synthesis contribute to the formation of the same types of compounds.[55]

Despite some early arguments to the contrary,[56] tholins are different materials than HCN polymers, although both contain considerable amounts of nitrile functional groups. Tholins appear to be, generally, better matches for extraterrestrial organic materials, both spectrally and with regard to their components. They are typically more C rich and N poor than HCN polymers, and have rather different mass distributions.[31]

Interestingly, room temperature hydrous spark discharge reactions are good analogs of hydrated Titan Tholins. Rather than mere serendipity, it seems likely that CH_4 and N_2, when excited, recombine to give very similar organic products, which hydrate similarly regardless of hydrolysis temperature. It also seems likely that among any one of these samples, multiple mechanisms of formation may be operative, as evidenced by isotopic labeling studies.[57] Regardless of the precise mechanism of synthesis, it does seem that there is a cohesive mechanism at some level in all of these syntheses.

Miller's pioneering work showed how easily compounds of biological relevance, for example urea, Gly, Ala, Asp, *etc.*, could be made in simple prebiotic simulations.[5] Consequently there has been a general trend to look for more compounds of biological relevance in these reactions. While many have been found, it must be borne in mind that organic structural isomerism leads to there being a very large number of compounds with identical formulas, even among the subset of those which are AAs (Table 3.3).

Although the excellent mass resolution and mass accuracy of FT-ICR-MS allow for unambiguous elemental formula assignment with minimal sample cleanup (for example, directly from organic solvent extraction[28, 35]), a single formula can represent many structures (Table 3.3), zwitterions may not electrospray well in either positive or negative mode, and ion suppression in such complex samples is likely. Additionally, compounds with the same molecular formula derived from the same isotopically-labeled starting materials can reasonably be expected to have similar isotopic enrichments. Thus, while FT-ICR-MS is an extremely powerful method for finding molecular formulas, it, alone, cannot provide unambiguous identification. As a result, further dimensions of analysis, such as liquid or gas phase separations, and fragmentation are required.

Table 3.3. Numbers of isomers for the 20 biologically encoded protein forming α-AAs.

AA	Molecular Formula	Number of Isomers		
		All[a]	No. of 3- or 4-rings	With α-AA Backbone[b]
Gly	$C_2H_5NO_2$	84	53	1
Ala	$C_3H_7NO_2$	391	244	1
Ser	$C_3H_7NO_3$	1391	857	2
Cys	$C_3H_7NO_2S$	3838	2422	2
Thr	$C_4H_9NO_3$	6836	4242	4
Asp	$C_4H_7NO_4$	65,500	25,036	14
Asn	$C_4H_8N_2O_3$	210,267	81,702	45
Pro	$C_5H_9NO_2$	22,259	8462	3 (6)[1]
Val	$C_5H_{11}NO_2$	6418	3973	2
Met	$C_5H_{11}NO_2S$	86,325	54,575	10
Glu	$C_5H_9NO_4$	440,821	172,617	71
Gln	$C_5H_{10}N_2O_3$	1,360,645	539,147	207
Leu, Ile	$C_6H_{13}NO_2$	23,946	14,866	4
Lys	$C_6H_{14}N_2O_2$	257,122	162,054	31
His	$C_6H_9N_3O_2$	89,502,542	13,563,099	902
Arg	$C_6H_{14}N_4O_2$	88,276,897	36,666,235	3563
Phe	$C_9H_{11}NO_2$	277,810,163	25,316,848	571 (6)[2]
Tyr	$C_9H_{11}NO_3$	2,132,674,846	209,838,248	8309 (43)[2]
Trp	$C_{11}H_{12}N_2O_2$	1,561,538,202,786	64,968,283,073	559,128 (1770)[2]

[a] "All" isomers denotes all structures which satisfy Lewis electron pairing rules for a given formula, the following column reports this number of isomers minus those structures, which include 3 or 4 membered rings.

[b] The total number of formula structural isomers that also contain the α-AA backbone motif. The numbers in parentheses refer to isomers which contain 1: a secondary α-AA backbone motif, or 2: a primary α-AA motif and an aromatic ring in the side-chain. Adapted from Meringer et al.[58]

All analytical methods have their limitations when analyzing AAs. AAs lacking chromophores are difficult to analyze *via* HPLC, and thus derivatization is typically required, commonly targeting amino groups.[59] While UV detection is typically robust, many extraneous compounds may absorb in near UV wavelengths that are typically monitored. Fluorescence detection overcomes this to some extent, as specific excitation and emission wavelengths allow peaks lacking the derivatization reagent, to be filtered out. Derivatization also renders AAs less polar, and more amenable to analysis *via* reverse phase chromatography.

GC-MS allows for simple detection and fairly unambiguous identification *via* electron impact fragmentation spectra, with numerous derivatization reagents allowing volatilization or polar compounds. One drawback, however, is that large polar compounds with multiple tags may not volatilize well, or they may thermally decompose before eluting from the GC column. On the positive side, fragmentation spectral libraries usually facilitate compound identification, but require comparison with authentic standards to further validate candidate compound identities.

UPLC-FD/TOF-MS is very sensitive, retention times are diagnostic, and a mass peak must correlate with a fluorescence peak. In other words, if there is no FD peak then the mass detected does not likely correspond to a primary amine. TOF-MS allows for good mass resolution, which means one can be fairly certain of molecular formula identification, and the addition of the OPA/NAC moiety constrains possible formulas. The downside is that obtaining fragmentation spectra requires more sophisticated hybrid Q-TOF instrumentation, and OPA/NAC does not tag some classes of compounds including 1,2-diamines (*e.g.* 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, and

ornithine) and secondary amines (*e.g.* proline and sarcosine). How similar these samples appear when a larger set of compounds is considered is the subject of ongoing work in our laboratories.

The UPLC-FD/TOF-MS techniques used here are very sensitive, highly unambiguous due to their multi-dimensionality, and are standardized and readily available, though they have blind spots, such as the inability to detect certain molecular weight amines of potential prebiotic interest, including secondary amines and vicinal diamines. Ideally, an LC-MS-compatible derivatization method should be developed that allows for separation and detection of primary, secondary and vicinal diamines and their enantiomers. The development of such derivatization techniques should be possible, and would be useful for future Solar System exploration programs.

3.5 Conclusions

Room temperature MU experiments conducted in the presence of water, with or without initial aqueous NH_3, hydrolyzed under various conditions, and numerous anhydrous Titan tholin hydrolysis experiments, produce many of the same AA products. These results demonstrate the ease with which chemical reactions in MU ED experiments can be extrapolated to simulated Titan conditions, to better understand monomer biomolecule, particularly AA, synthesis under possible extraterrestrial environmental scenarios. However, we note that considerable care must be exercised in the identification of these target analytes, as neither chromatographic retention time, nor mass analysis, alone, are fully reliable detection techniques. Our results suggest there may be a remarkable cohesiveness in the types of AAs that can be produced in various primitive planetary environments given a reducing atmosphere.

3.6 Acknowledgements

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CHAPTER 4: A PLAUSIBLE SIMULTANEOUS SYNTHESIS OF AMINO ACIDS AND SIMPLE PEPTIDES ON THE PRIMORDIAL EARTH

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4.1 Abstract

Original, archived samples from an additional Stanley Miller experiment were recently discovered. This experiment, performed in 1958, was designed to study AA polymerization under simulated early Earth conditions. Miller sparked a gas mixture of CH_4, NH_3, and H_2O, while intermittently adding the plausible prebiotic condensing reagent cyanamide. For unknown reasons, an analysis of the samples was not previously reported. We analyzed the archived samples for AAs, dipeptides, and diketopiperazines using a combination of liquid chromatography, ion mobility spectrometry, and high resolution mass spectrometry. A dozen AAs, 10 glycine-containing dipeptides, and 3 glycine-containing diketopiperazines were detected. Miller's experiment was repeated to compare the analysis of freshly prepared samples with that of samples from the original cyanamide experiment. Upon analyzing samples from the repeated cyanamide experiment, similar polymerization products were detected to those observed in the original samples, corroborating the findings from the analysis of Miller's archived cyanamide experimental samples. Furthermore, the mechanism of the cyanamide-mediated AA polymerization chemistry was elucidated by conducting aqueous solution heating experiments. These explorations indicated that the intermediates of the Strecker synthesis of AAs, the amino acid amide and aminonitrile, play key roles in facilitating the

observed polymerization. The results of this work highlight the potential importance of condensing reagents to have generated diversity within the prebiotic chemical inventory.

4.2 Introduction

A combination of Miller's pioneering 1953 experiment[1] and the subsequent findings of extraterrestrial organic compounds in meteorites[2-4] indicates that the synthesis of prebiotic organics thought to be necessary for the origin of life is a robust process, both on the primitive Earth and other planetary bodies.[5, 6] However, the transition from simple molecules, such as AAs, to more complex ones, such as peptides, has proven challenging under plausible primordial conditions. Although hydrothermal vent and comet-impact based syntheses of peptides have been reported, questions remain about their plausibility under prebiotic geochemical conditions.[7] In addition, concentrated salts, clays and Cu^{2+} have been suggested as being important AA condensation reagents,[8, 9] although these have not been demonstrated to be effective polymerization agents under the natural geochemical environments that may have existed on the early Earth. For example, Cu^{2+} in the primitive oceans would have been in the form of Cu^{1+} and its concentration would have been very low because of the presence of HS^-.[10] Additionally, other reduced metal ions, such as Fe^{2+}, may have been present[11] and could have played a role in shaping prebiotic environments and the chemical reactions that may have occurred therein. Other potential prebiotic polymerization agents, such as carbonyl sulfide (COS), have been proposed,[12] although the overall efficiency with respect to the variety of AAs that can undergo polymerization with this reagent has not been explored.

In carbonaceous meteorites the timescale for AA production is typically estimated to be between 10^3 and 10^6 years,[13] and could be as short as 1-10 years.[14] The prebiotic

chemistry that took place on the meteorite parent bodies during the aqueous alteration phase is considered to have produced mainly simple monomeric compounds[5] and complex, poorly characterized polymers.[15, 16] Only trace quantities of glycine dipeptide and its diketopiperazine (DKP) have been detected in a few cases.[17] **Figure 4.1.a** provides examples of the structural differences between linear dipeptides and DKPs.

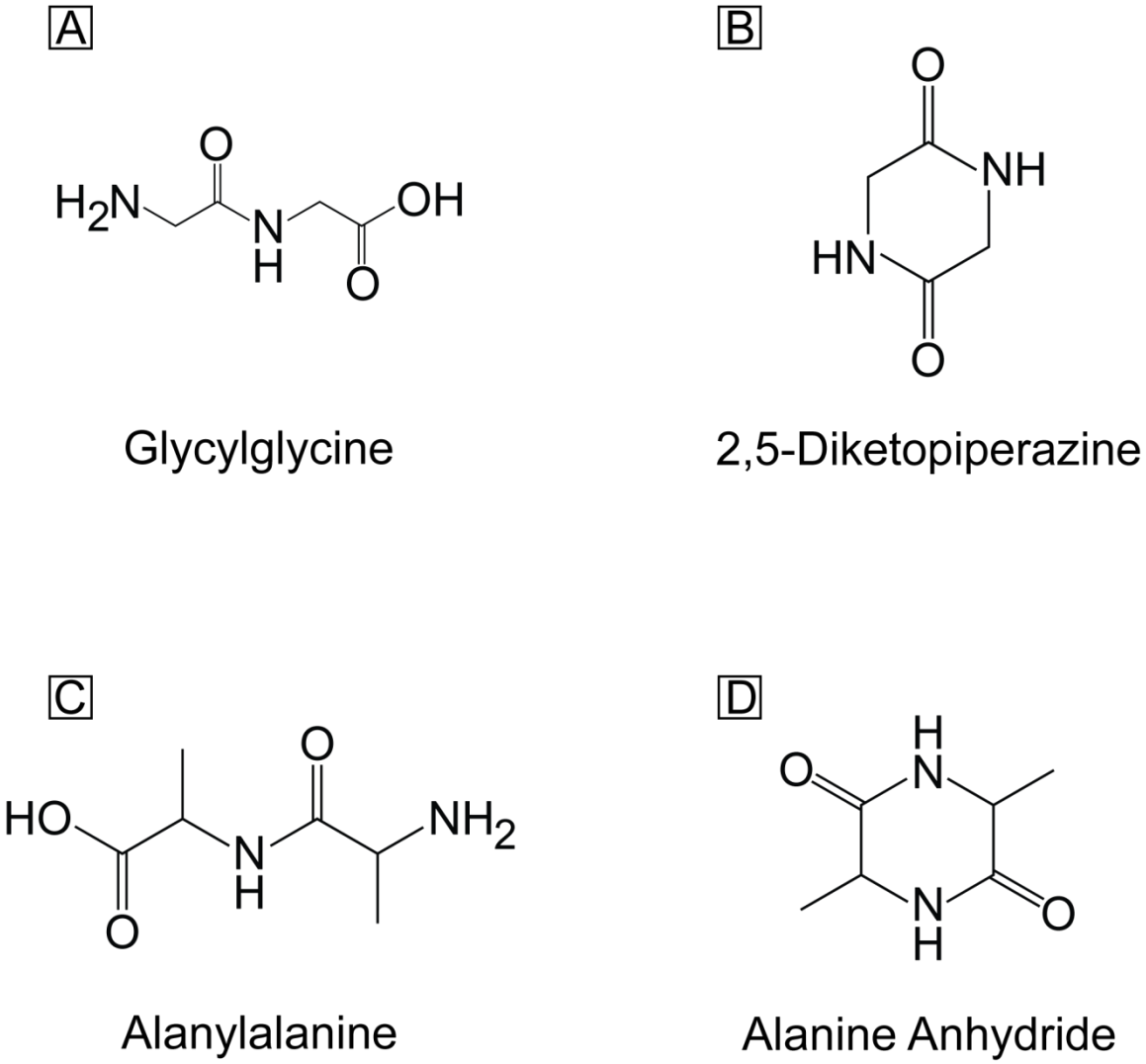

Figure 4.1. The glycine dipeptide (A), glycine DKP (B), alanine dipeptide (C), and alanine DKP (D) are shown as examples of the structural differences between linear and cyclic dipeptides.

Recently, archived, stored portions of the solutions from experiments Miller carried out in 1958 were found.[18] Included in this set were labeled vials from an experiment in which reduced gases (CH_4 and NH_3) were subjected to a spark discharge for ~7 days and over the course of the experiment, cyanamide was intermittently added to the aqueous phase. These archived cyanamide samples were part of a large collection of portions that Miller saved from a number of his experiments in the 1950s.[5] For unknown reasons, Miller never performed a chemical analysis of the products of the cyanamide experiment and others conducted in 1958.[18]

While it has not yet been demonstrated that cyanamide can be formed within ED experiments, the production of cyanamide in plausible prebiotic conditions comprised of CH_4, NH_3, H_2O, and UV light was reported nearly 50 years ago, and was proposed to be a possible prebiotic condensing reagent.[19, 20] Preliminary experiments supported this scenario,[21] although the reaction seemed to be most favorable at acidic pH values less than the pK_1 of the AA.[22] This suggested that the reactive AA species is H_3N^+-C(RR′)-COO^- (R and R′ representing the AA side chains). It has also been proposed that cyanamide can activate N-acyl α-AAs to form a 5(4H)-oxazolone intermediate that can help facilitate the coupling of sterically hindered α-AAs.[23] In addition, cyanamide has been suggested to be influential in other important prebiotic reactions, such as the synthesis of activated pyrimidine ribonucleotides[24] and 2'-deoxynucleotides.[25]

Miller never carried out a detailed analysis of his 1958 cyanamide experiment, but he did measure the absorption at 280 nm when he collected various fractions during chromatographic separation of the discharge solution from the cyanamide experiment and found absorption in several samples where peptides were expected to elute.[26, 27] We have

now analyzed the 1958 cyanamide spark discharge residues to ascertain if both AAs and simple peptides had actually been synthesized simultaneously in this prebiotic simulation experiment. In addition, Miller's cyanamide experiment was repeated and analyzed for peptides to determine what impact, if any, storage conditions could have had on the products detected in the original samples. Lastly, aqueous solution heating experiments were performed to investigate the interaction between cyanamide and amino acids.

4.3 Materials and Methods

4.3.1 *Chemicals and Reagents*

The chemical analyses discussed in this study were performed either at the NASA Goddard Space Flight Center (GSFC) or the Georgia Institute of Technology (GT). All glassware and sample handling tools used at GSFC and GT were thoroughly cleaned using polished water and then wrapped in aluminum foil and heated overnight, in air, at 500 °C. The GSFC analyses used Millipore Integral 10 water (18.2 MΩ cm, 3 ppb total organic carbon), while the GT analyses used Nanopure Diamond water (18.2 MΩ cm). The reagents used at GSFC were purchased from Sigma-Aldrich, or Fischer Scientific. Standard AA solutions (~10^{-4} M) were prepared by dissolving individual AA crystals (97-99 % purity) in water before being combined to allow for their measurement in a single chromatographic separation (GSFC analyses). Unless otherwise stated, all chemicals used at GT were purchased from Sigma-Aldrich, Chem-Impex International, Bachem Bioscience, or EMD Chemicals, with known purities ranging from 98 % to 99+ %. Stock dipeptide and DKP standard solutions (~10^{-5} M) were freshly prepared by dissolving individual dipeptide and DKP crystals in Nanopure water prior to being mixed (GT analyses).

High temperature acid vapor hydrolysis (GSFC analyses) used 6 M doubly-distilled hydrochloric acid (ddHCl).[28] Liquid phase acid hydrolysis (GT analyses) used analytical grade HCl. Cation exchange chromatography (GSFC analyses) was performed using 2 M NaOH, 1.5 M ddHCl, and 2 M NH_4OH. The 2 M NH_4OH was prepared from NH_3 gas, while the 2 M NaOH was prepared by dissolving 32 g of NaOH in 0.4 L water.

Three reagents were prepared for derivatization of samples prior to AA analysis at GSFC: (1) 0.1 M sodium borate, (2) OPA/NAC, and (3) double distilled (dd) 0.1 M hydrazine hydrate. Reagents were prepared as follows: (1) was prepared by dissolving solid sodium borate, after heating at 500 °C for 3 hours, in Millipore water; reagent (2) was prepared by dissolving 4 mg OPA in 300 µL Fisher Optima grade methanol, and mixing with 670 µL of 0.1 M sodium borate (pH 9) and 30 µL of 0.5 M NAC, and (3) was prepared as described by.[29]

Three reagents were used for LC-MS AA analysis of original samples at GSFC: (A) water, (B) methanol (Fisher Optima grade), and (C) 50 mM NH_4Fo (adjusted to pH 8), with 8% methanol.[29] Two reagents were used for dipeptide and DKP analysis of the original cyanamide samples at GT: (A) 10 mM ammonium acetate (NH_4OAc) and (B) LC-MS grade acetonitrile. Reagent (A) was prepared by dissolving 770 mg of NH_4OAc crystal into 1 L of water. Two reagents were used for analysis of repeated cyanamide experimental samples and heating experiment samples: (A) 10 mM NH_4Fo and (B) LC-MS grade acetonitrile. Reagent (A) was made by dissolving 631 mg of NH_4Fo crystal into 1 L of water.

4.3.2 *Sample Discovery and Experimental Setup*

Miller's preserved samples were discovered in 2007 at the Scripps Institution of Oceanography, where Miller had stored samples from his original 1950s spark discharge experiments. Miller's original laboratory notebooks indicate that the cyanamide spark discharge experiment was conducted in 1958, using a version of his original spark discharge apparatus, described elsewhere,[30] prior to collecting and saving the samples.[27] The initial experimental conditions included 300 mmHg CH_4, 250 mmHg NH_3, and 250 mL of water, and the experiment was started by generating a spark discharge across two tungsten electrodes, using a Tesla coil, and gently boiling the water.[27] Information regarding the type of Tesla coil used by Miller for his ED experiments, and its output, can be found elsewhere.[31] The experiment ran for 17.5 hours before being stopped for 6 hours to allow for the introduction of 3.3 mg of cyanamide in 50 mL of water. The experiment was stopped by turning off the Tesla coil and removing the apparatus from the heat source. After the addition of cyanamide, the experiment was then resumed for another 12.5 hours. The experiment was restarted by turning the Tesla coil back on and without boiling the water. For the remainder of the experiment, the use of heat was discontinued, perhaps to minimize the risk of thermally decomposing cyanamide. The experiment was then stopped for 5 hours for a second addition of cyanamide (4.4 mg of cyanamide in 25 mL of water). Afterward, the experiment ran for another 29 hours before being stopped a third time to attempt a final introduction of 4.4 mg of cyanamide in 25 mL of water. However, Miller noted that he "could not pump ligand back into the apparatus" because of pressure build-up inside the reaction flask and as a result removed 1 L of gas from the apparatus to allow the experiment to continue without over-

pressuring the apparatus, meanwhile saving 500 mL of the extracted gas.[27] The experiment was resumed after 4.5 hours, and ran for another 86 hours, before being terminated by turning off the Tesla coil. An overview of this experimental design is provided in Table 4.1.

Table 4.1. Time sequence of Miller's cyanamide experimental design noting the duration of the experiment and the details of cyanamide introduction into the reaction flask.

Time (hours)	To Be Introduced Into Reaction Flask	Action
0	250 mL H_2O, 300 mmHg CH_4, 250 mmHg NH_3	Start spark, start heat
17.5	3.3 mg cyanamide in 50 mL H_2O	Stop spark, stop heat
23.5	-	Start spark, no heat
36	4.4 mg cyanamide in 25 mL H_2O	Stop spark
41	-	Start spark, no heat
70	4.4 mg cyanamide in 25 mL H_2O	Stop spark
74.5	-	Start spark, no heat
160.5	-	Stop spark

Miller first used IEC to isolate total AAs, followed by subsequent separation of individual sample fractions using acid cation exchange chromatography.[26, 31] Sample fractions were then evaporated until dry and archived until their discovery and analyses.

4.3.3 *Original Miller Cyanamide Sample Analyses*

The use of the OPA/NAC reagent coupled with sophisticated LC-MS techniques has proven to be a powerful approach for analyzing primary AAs and amines in the old Miller extracts.[18, 32] Dipeptides in the samples can be readily characterized by a combination of liquid chromatography, ion mobility spectrometry (IMS), and high resolution tandem mass spectrometry techniques.

AAs were analyzed at GSFC using HPLC-FD/QqQ-MS. Dipeptides and DKPs were analyzed at GT using ultra performance liquid chromatography coupled to

quadrupole-travelling wave ion mobility spectrometry-time of flight mass spectrometry (UPLC-Q-TWIMS-TOF-MS).

Aliquots of Miller's original cyanamide samples were prepared for AA analysis, wherein half of each sample aliquot underwent acid hydrolysis, while the other half did not. Both the hydrolyzed and unhydrolyzed fractions were desalted.[6] AA data reported here were obtained from the analyses of the unhydrolyzed fractions. Prior to analysis, 10 µL of each sample solution were mixed with 10 µL of 0.1 M sodium borate, 5 µL of OPA/NAC, and derivatized for 15 minutes.[33] Once complete, the derivatization reaction was then quenched with 75 µL of 0.1 M hydrazine hydrate and immediately analyzed by HPLC-FD/QqQ-MS.

AA analysis was carried out using a Waters 2695 HPLC coupled to a Waters 2475 fluorescence detector and a Waters Quattro Micro API triple quadrupole mass spectrometer. Chromatography was performed using a 250 mm x 4.6 mm, 5 µm particle size Phenomenex Luna phenyl-hexyl column. The following mobile phases were used for chromatographic separation: (A) Millipore water, (B) methanol (Optima grade), and (C) 50 mM NH_4Fo (pH 8) with 8% methanol. The fluorescence detector utilized an excitation wavelength of 340 nm and an emission wavelength of 450 nm. For chromatographic separation of target analytes, the following gradient was used: 0-5 min, 100 % C; 5-15 min, 0-83 % A, 0-12 % B, 100-5 % C; 15-22 min, 83-75 % A, 12-20 % B, 5 % C; 22-35 min, 75-35 % A, 20-60 % B, 5 % C; 35-37 min, 35-0 % A, 60-100 % B, 5-0 % C; 37-45 min, 100 % B; 45-46 min, 100-0 % B, 0-100 % C 46-55 min, 100 % C. The buffer flow rate used was 1 mL min^{-1} and the flow was split 90 % to the fluorescence detector and 10 % to the mass spectrometer. The QqQ-MS was operated in Q_1 scan mode, with a scan

speed of 450 amu sec^{-1}. An ESI source operating in positive ion mode was used to detect OPA/NAC AA derivatives in the 50-500 m/z range. The ESI settings used were the following: desolvation gas (N_2) temperature/flow rate: 350°C/650 L hr^{-1}, respectively; capillary voltage: 3.8 kV; cone voltage: 30 V.

A portion of Miller's original cyanamide spark discharge sample residues were dissolved in 500 µL of a 50:50 (v:v) mixture of methanol and water prior to direct dipeptide and DKP analysis by UPLC-Q-TWIMS-TOF-MS. Aliquots (50 µL) of the re-suspended samples were dried under nitrogen in preparation for liquid-phase acid hydrolysis as described elsewhere.[34] Afterward, any remaining HCl was evaporated with a dry nitrogen flow. The acid hydrolyzed sample residues were finally reconstituted in 50 µL of a 50:50 (v:v) mixture of methanol and water before analysis. Hydrolyzed sample extracts were used for comparison to samples that were analyzed directly, to determine if the peptides tentatively identified in the original samples were decomposed by hydrolysis, which would be indicative of the breaking of the peptide bond.

A Waters ACQUITY UPLC coupled to a Synapt G2 high definition mass spectrometer operating in positive ion mode (Waters, Milford, MA, USA) was used for dipeptide and DKP analyses. Chromatographic separations were performed using a 100 mm x 2.1 mm, 1.7 µm particle size Waters ACQUITY UPLC BEH amide HILIC column. The mobile phases used were (A) 10 mM NH_4OAc and (B) LC-MS grade acetonitrile. The eluent flow rate was 0.3 mL min^{-1}. A typical chromatographic gradient used was: 0-4 min, 100 % B; 4-5 min, 100-96 % B; 5-10 min, 96-45 % B; 10-13.5 min, 45-10 % B; 13.5-14.5 min, 10 % B; 14.5-15 min, 10-100 % B; 15-16 min, 100 % B. Column temperature was maintained at 60 °C and samples held at 5 °C prior to analysis.

Peptides were identified following a two-step procedure. First, accurate mass survey UPLC-MS runs were screened against a custom-built MarkerLynx (v.4.1) database of accurate masses to identify peaks that matched dipeptide $[M+H]^+$ ions. A match tolerance of 10 mDa was used. The custom database was created by inputting dipeptide accurate masses calculated in Excel using SQLite database browser v.1.3. AAs considered in potential dipeptides included asparagine, glutamic acid, serine, glycine, alanine, amino-*n*-butyric acid, α-aminoisobutyric acid, valine, glutamine, proline, threonine, arginine, histidine, lysine, leucine, isoleucine, phenylalanine, tryptophan, tyrosine, aspartic acid, and ornithine. Following this screening step, MS/MS experiments were conducted via quadrupole precursor ion selection, followed by TWIMS ion mobility gas-phase separation, and transfer-cell collision-induced dissociation (CID) to fragment the peptides tentatively identified in the screening step. The experiments involved acquiring dipeptide product ion spectra in a retention time window of 0.5 minutes centered on the retention times observed in UPLC-TWIMS-MS runs carried out without precursor ion selection. Various energies were selected for CID experiments to ensure observing diagnostic fragment ions (e.g. y_1). These optimum energies were determined *a priori* using dipeptide standards. MS/MS experiments helped optimize the duty cycle of the quadrupole by allowing it to focus on select precursor ions, as opposed to continuously cycling through a list of potential precursor targets. The TWIMS trap cell utilized a helium gas flow of 190 mL min^{-1}, while the main mobility cell used a nitrogen gas flow of 100 mL min^{-1}. The wave velocity was ramped from 700 to 2000 m s^{-1}, and the wave height from 22 to 30 V. Peptides were identified by elemental formula, and *de*

novo sequencing using PEAKS software (v. 5.3 Bioinformatics Solutions, ON, Canada). A tolerance of 10 mDa was used for matching fragment ions.

A dual ESI system was utilized for lock mass correction purposes. The primary ESI source was used and operated according to the following conditions: capillary voltage, 4 kV; sample cone voltage, 20 V; extraction cone voltage, 4 V; source temperature, 120 °C; desolvation gas (N_2) temperature, 250 °C; desolvation gas flow rate, 650 L hr^{-1}. A 0.5 mM sodium formate solution was used for daily calibration of the mass spectrometer. The calibration solution was prepared by mixing 1000 µL of 0.1 M NaOH, 900 µL of deionized water and 100 µL of formic acid. The calibration solution was then diluted to 20 mL using deionized water. Because minor drifts in the m/*z* scale may occur after initial calibration, a reference ESI source was used to provide an independent signal using leucine-enkephalin as the standard. The reference ESI source had the same settings as the main one, except that it used a capillary voltage of 2.8 kV. The TOF analyzer was operated in "V-optics mode", which utilized a reflectron to supply a full width at half maximum mass resolution of 21,000 based on the protonated ion of leucine-enkephalin at m/*z* 556.2771. The detector setting was 2575 V. The range acquired in all experiments was 50-1200 m/*z*.

4.3.4 *Repeated Cyanamide Experiment Design*

Duplication of Miller's cyanamide experiment was based on his original laboratory notebooks.[27] The repeated experiment used heat and a spark in alternating one hour on/off cycles to preserve the lifetime of the spark generator and to minimize condensation of water vapor on the electrode tips, thereby mitigating the possibility of quenching the spark. Because of this cyclical sparking pattern, the time period over

which the experiment was run was doubled to allow for an equivalent amount of spark reaction time as in the original cyanamide experiment. The initial experimental conditions were 250 mL of water, 300 mmHg CH_4 and 250 mmHg NH_3, all inserted into a 3 L reaction flask equipped with 2 tungsten electrodes. A spark was generated across the electrodes over a 14 day period. The times at which cyanamide was introduced into the reaction flask were also determined with the cyclical sparking pattern, and Miller's original protocol, in mind. General procedures used to prepare the reaction flask for the repeated cyanamide experiment were based on the protocol outlined by Parker et al.[35]

An aqueous cyanamide solution was introduced into the 3 L reaction flask at three distinct times over the course of the experiment. To facilitate each cyanamide addition, the reaction was stopped by removing the apparatus from the heat and the spark for the same amount of time as Miller had originally done (Table 4.1). However, Miller's notebooks did not specify how the cyanamide introduction was performed; therefore an introduction apparatus was designed and implemented to perform this task (Figure 4.1). Prior to each introduction, the aqueous cyanamide solution was loaded into a separatory funnel, modified to be equipped with a cooling jacket, which was simultaneously connected to a vacuum system. A dry ice/acetone bath was placed in the cooling jacket surrounding the separatory funnel. The purpose of this cooling bath was to freeze the cyanamide solution and force dissolved gases out of the aqueous phase and into the above headspace of the separatory funnel. The headspace gas above the frozen solution was then evacuated and replaced with argon gas and evacuated again. The solution was then allowed to thaw at room temperature, prior to undergoing a second round of freezing, degassing, and thawing as described above. Once thoroughly degassed, the aqueous

cyanamide solution was introduced into the reaction apparatus using a sufficient pressure of Ar gas to push the aqueous solution into the reaction flask. Argon gas was chosen for the aforementioned uses, as opposed to alternative gases like N_2, because argon is chemically inert and because the use of argon would make it possible to avoid introducing any additional quantities of elements that were part of the initial experimental conditions (i.e. nitrogen).

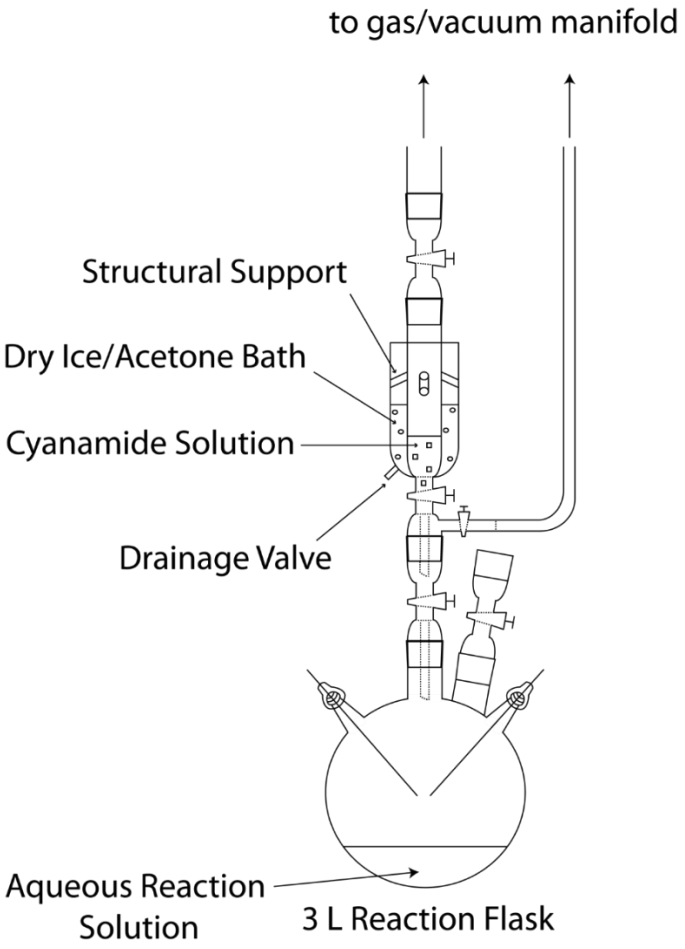

Figure 4.2. Schematic diagram of the setup used to conduct aqueous cyanamide introductions into the reaction apparatus without compromising the seal of, or introducing contaminants into, the flask while repeating Miller's cyanamide spark discharge experiment.

The first cyanamide addition came at t = 35 hours, where 3.3 mg of cyanamide dissolved in 50 mL H_2O was introduced into the 3 L reaction flask. The experiment was stopped for 6 hours for this cyanamide addition before restarting the experiment at t = 41 hours by resuming sparking and discontinuing heating the apparatus. The second aliquot of cyanamide was added at t = 66 hours by introducing 4.4 mg of cyanamide dissolved in 25 mL H_2O. The experiment was discontinued for 5 hours for this introduction before being resumed at t = 71 hours. The third and final cyanamide addition was at t = 129 hours where 4.4 mg of cyanamide dissolved in 25 mL H_2O was introduced. The experiment was resumed at t = 133.5 hours and then ran uninterrupted until the experiment ended at t = 305.5 hours. Liquid sample was carefully collected from the 3 L reaction flask, placed in sterilized receptacles, and stored in a freezer prior to analysis.

In addition to repeating Miller's cyanamide experiment, control and blank experiments were conducted for comparison of analytical results to the repeated cyanamide experiment. The control and blank experiments were carried out in a fashion similar to the repeated cyanamide experiment, with specific differences. The control experiment differed from the repeated cyanamide experiment in that instead of introducing aqueous cyanamide solutions, only Nanopure water was introduced in volumes that matched those introduced during the repeated cyanamide experiment. This was done to determine if dipeptides could be detected without the presence of cyanamide. In the blank experiment, carbon was not introduced into the reaction flask. Instead, 300 mmHg N_2 was introduced, as opposed to 300 mmHg CH_4. Additionally, Nanopure water was introduced as was done for the control experiment, as opposed to aqueous cyanamide solutions.

4.3.5 *Repeated Cyanamide Experiment Sample Analyses*

Samples from the repeated cyanamide ED experiment were analyzed UPLC/QqQ-MS for confirmation of AA polymerization results. Samples were prepared by drying down an aliquot of the discharge solution and reconstituting it in a 50:50 (v:v) mixture of LC-MS grade acetonitrile and 10 mM NH_4Fo equal in volume to that of the sample prior to the dry-down step. The supernatant was then extracted for analysis. Concentrated samples were also prepared and analyzed. To concentrate the samples, aliquots (20 mL) of the repeated cyanamide experiment samples were lyophilized overnight. The residues produced from this freeze-drying process were reconstituted in 2 mL of a 50:50 (v:v) mixture of LC-MS grade acetonitrile and 10 mM NH_4Fo. The resulting supernatant was then sampled for analysis.

UPLC/QqQ-MS experiments were performed with an Agilent 1290 Infinity UPLC unit coupled to an Agilent 6430 QqQ-MS operated in multiple reaction monitoring (MRM) mode. Chromatography was carried out with a 100 mm x 2.1 mm, 1.7 µm particle size Waters ACQUITY UPLC BEH amide HILIC column. The mobile phases used were A) 10 mM NH_4Fo and B) acetonitrile (LC-MS grade). Linear dipeptides were eluted using isocratic runs of 30 % B. Isocratic elution of DKPs was achieved with a mobile phase composition of 85% B. The mobile phase flow rate was 0.3 mL min^{-1}, while the column was maintained at a temperature of 60 °C. The autosampler tray was kept at a temperature of 5 °C, the injection volume used was 5 µL, and the run times were 3 minutes. The QqQ-MS was equipped with an ESI source that was operated in positive mode, with a capillary voltage of 4 kV, nebulizing gas (N_2) pressure of 15 psi, and a desolvation gas temperature/flow rate of 300 °C/6 L min^{-1}, respectively.

4.3.6 *Heating Experiment Design*

Aqueous solutions were prepared for heating experiments designed to evaluate the polymerization of AAs under a variety of pH regimes and a constant temperature. The temperature used for the experiments was 50°C and the pH ranges across several experiments were: a) pH 1-2, b) pH 6-7, c) pH 9-10, and d) pH 12-13. The pH 1-2 solutions were made of 0.1 M ddHCl. The pH 6-7 solutions were made by mixing 0.1 M potassium dihydrogenorthophosphate with 0.1 M potassium hydroxide (KOH) until a pH between 6 and 7 was achieved. The KOH used was of 91.3% purity in KOH (NOAH Technologies Corporation, San Antonio, Texas). The pH 9-10 solutions were made of 0.01 M sodium tetraborate decahydrate. The pH 12-13 solutions were made by mixing 0.1 M KOH with 0.1 M ddHCl until a pH between 12 and 13 was achieved. To each solution, glycine and either cyanamide, or dicyandiamide were added until the solution was 0.01 M in glycine and 0.05 M in cyanamide, or dicyandiamide. For each of the four tested pH ranges, control samples were created that contained 0.01 M glycine, but did not contain a condensing reagent.

To better simulate the chemical environment contained within the discharge apparatus, heating experiment solutions were prepared in addition to those described above. To the stock pH 9-10 solutions, NH_3 was added in the form of NH_4Cl, until the solution became 0.01 M in glycine, 0.05 M in either cyanamide or dicyandiamide, and 0.01 M in NH_4Cl. Furthermore, a set of samples was prepared from the pH 9-10 stock solutions of glycine, glycine + cyanamide, and glycine + dicyandiamide, to contain 0.01 M glycinamide·HCl, or 0.01 M glycinonitrile·HCl. After the addition of glycinamide·HCl and glycinonitrile·HCl, these samples had a pH of ~8.5-9.

4.3.7 *Heating Experiment Sample Analyses*

Aliquots of each solution were analyzed immediately upon being made, thereby representing samples with 0 days of heating. Sample portions that underwent heating were sampled periodically over the course of 3 weeks to evaluate the temporal variability of the polymerization chemistry. Once collected, each heated sample was dried down and re-suspended in a 50:50 (v:v) solution of LC-MS grade acetonitrile and 10 mM NH_4Fo before analysis. Heated samples were analyzed for peptides using the retention times of parent masses associated with peptides of interest, as well as detection of corresponding fragment ions, as was similarly outlined for the repeated experiment samples.

4.4 Results and Discussion

Analyzing Miller's archived samples resulted in the detection of 12 AAs, 10 glycine-containing dipeptides, and 3 glycine-containing DKPs (Table 4.2).

Table 4.2. AAs, dipeptides, and DKPs identified and quantified in this study.

AAs[a]	Dipeptides[b]	DKPs
Glycine	Gly-Gly	Cyclo(Gly-Gly)
Alanine	Gly-Ala	Cyclo(Gly-Pro)
β-Alanine	Gly-Thr	Cyclo(Leu-Gly)
Serine	Gly-Pro	
α-Aminobutyric Acid	Pro-Gly	
β-Aminobutyric Acid	Gly-Val	
γ-Aminobutyric Acid	Val-Gly	
Aspartic Acid	Gly-Glu	
Glutamic Acid	Glu-Gly	
Valine	Leu-Gly	
Isovaline		
Isoleucine		

[a] Additional AAs were tentatively identified, but were not quantitated and therefore not included here, but are listed in Table 4.3. [b] Additional dipeptides, as well as higher order peptides, such as the tripeptides Pro-Pro-Gly and Asp-Asp-Gly, were tentatively identified within the archived samples, but are not included here because they were not quantified. These initial identifications indicate that the formation of tri- and higher peptides in prebiotic simulation experiments warrants further investigation.

4.4.1 *AAs in the Original Miller Cyanamide Samples*

The AAs produced by the cyanamide experiment were synthesized at relatively high yields, and when compared to those detected in Miller's classic and volcanic,[32] and hydrogen sulfide-containing[18] spark discharge experiments, are present in similar relative abundances (Figure 4.2). Major AAs with chiral centers (e.g. aspartic and glutamic acids, serine, alanine, and isovaline) were racemic (D/L ~ 1) within error (10 %), indicating their abundances were minimally influenced by terrestrial L-AA contamination during sample storage and processing. A comprehensive list of AAs detected by both chromatographic and mass spectral determination is provided in Table 4.3.

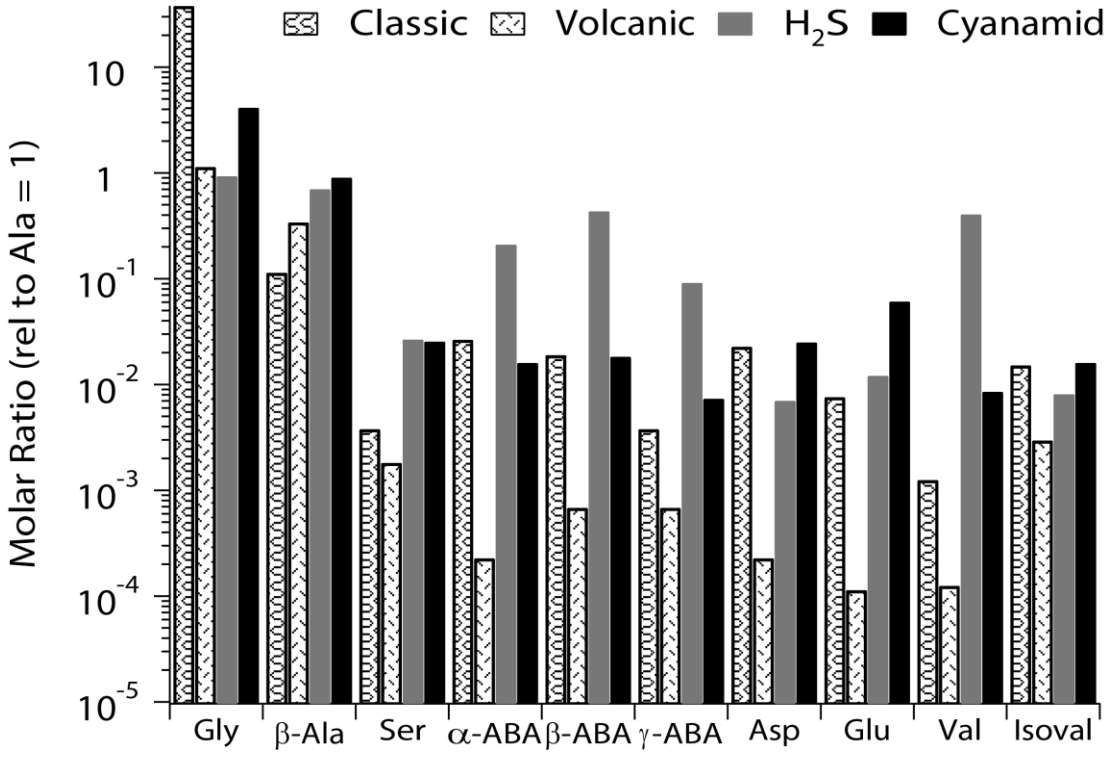

Figure 4.3. Molar ratios (relative to D+L-Ala = 1) of major AAs in unhydrolyzed samples from Miller's classic, volcanic, H_2S, and cyanamide spark discharge experiments.

Table 4.3. AAs detected in the archived samples. Relative abundances are normalized to alanine as opposed to glycine because the heavier alanine is farther away from the low molecular weight cut-off of the various MS techniques used in this study and is thus detected with higher sensitivity, thereby providing for more reliable relative quantitation.

AA	STD LC RT (min)	Sample LC RT (min)	OPA/NAC Derivatized Mass $[M+H]^+$	STD Mass RT (min)	Sample Mass RT (min)	Molar Abundance (relative to Ala = 1)
D-Asp	3.52	3.60	395	3.53	3.61	2.45×10^{-2}
L-Asp	3.79	3.90	395	3.83	3.89	
L-Glu	5.40	5.58	409	5.37	5.51	5.96×10^{-2}
D-Glu	5.73	5.88	409	5.75	5.90	
D-Ser	11.84	11.81	367	11.80	11.82	2.49×10^{-2}
L-Ser	12.09	12.07	367	12.06	12.08	
Gly	15.06	15.04	337	15.02	15.00	4.05
β-Ala	15.62	15.62	351	15.58	15.58	0.88
D-Ala	16.44	16.43	351	16.39	16.43	1
L-Ala	16.72	16.71	351	16.69	16.69	
γ-ABA	16.44	16.43	365	16.41	16.36	7.16×10^{-3}
D-β-ABA	17.09	17.08	365	17.06	17.08	1.79×10^{-2}[a]
L-β-ABA	17.35	17.35	365	17.32	17.32	
D/L-α-ABA	17.83	17.83	365	17.78	17.78	1.57×10^{-2}
D-Iva	17.92	17.91	379	17.89	17.89	1.57×10^{-2}
L-Iva	18.00	18.00	379	17.96	17.96	
L-Val	18.12	18.14	379	18.35	18.37	8.36×10^{-3}
D-Val	18.39	18.39	379	18.35	18.35	
D-Ile	18.94	18.94	393	18.91	18.91	8.24×10^{-4}
L-Ile	19.42	19.40	393	19.39	19.35	-
α-AIB[b]	17.35	17.35	365	17.32	17.32	-
D-Ise[b]	-	13.83	367	-	13.84	-
L-Ise[b]	-	14.32	367	-	14.30	-

[a] L-β-ABA and α-AIB interfered with one another during HPLC-FD/QqQ-MS analysis and were not quantitated. Due to the nearly racemic nature of major AAs, the molar abundance of β-ABA is estimated here as twice the abundance of D-β-ABA.
[b] AAs tentatively detected, but not quantified.

4.4.2 *Dipeptides in the Original Miller Cyanamide Samples*

Glycine-containing dipeptides and DKPs were targeted for analysis. Glycine is the simplest AA and one of the most abundant AAs formed by ED experiments. Therefore, many peptides present in the samples reported here should contain glycine. Multiple analysis workflows were used to confirm the identity and quantity of dipeptides and DKPs. Figure 4.3 shows an example of how dipeptides were preliminarily detected using accurate mass UPLC-MS analysis to screen for specific dipeptide $[M+H]^+$ ions based on retention time and high resolution exact mass, compared to standards.

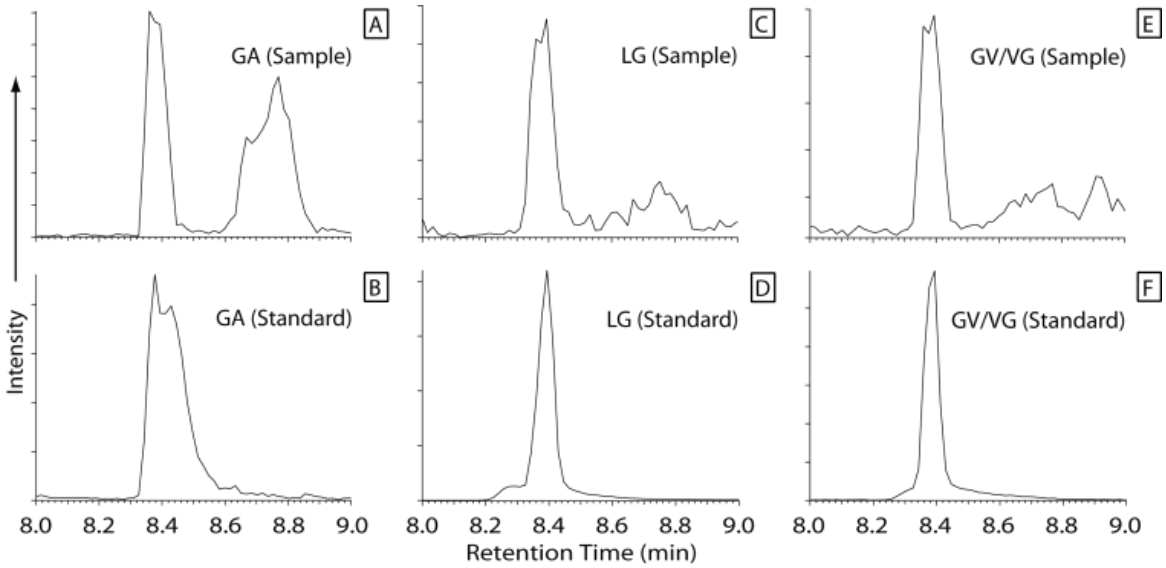

Figure 4.4. Shown here is an example of how glycine-containing dipeptides were initially detected. Extracted UPLC mass chromatograms, corresponding to the 8-9 minute retention time window are displayed for specific dipeptides detected in Miller's sample fraction 57-67, and for standard traces. Chromatograms A) and B) were extracted by screening the total ion chromatograms for m/z 147.0770, C) and D) correspond to m/z 189.1239, and E) and F) are associated with m/z 175.1083. Note: GA = glycylalanine, LG = leucylglycine, and GV/VG = glycylvaline/valylglycine.

After initial dipeptide $[M+H]^+$ screening, employing UPLC-Q-TWIMS-TOF-MS allowed for unambiguous identification and quantitation of the targeted dipeptides

(Figure 4.4). A complete list of glycine-containing dipeptides (Table 4.4) and DKPs (Table 4.5) detected in the original samples are provided.

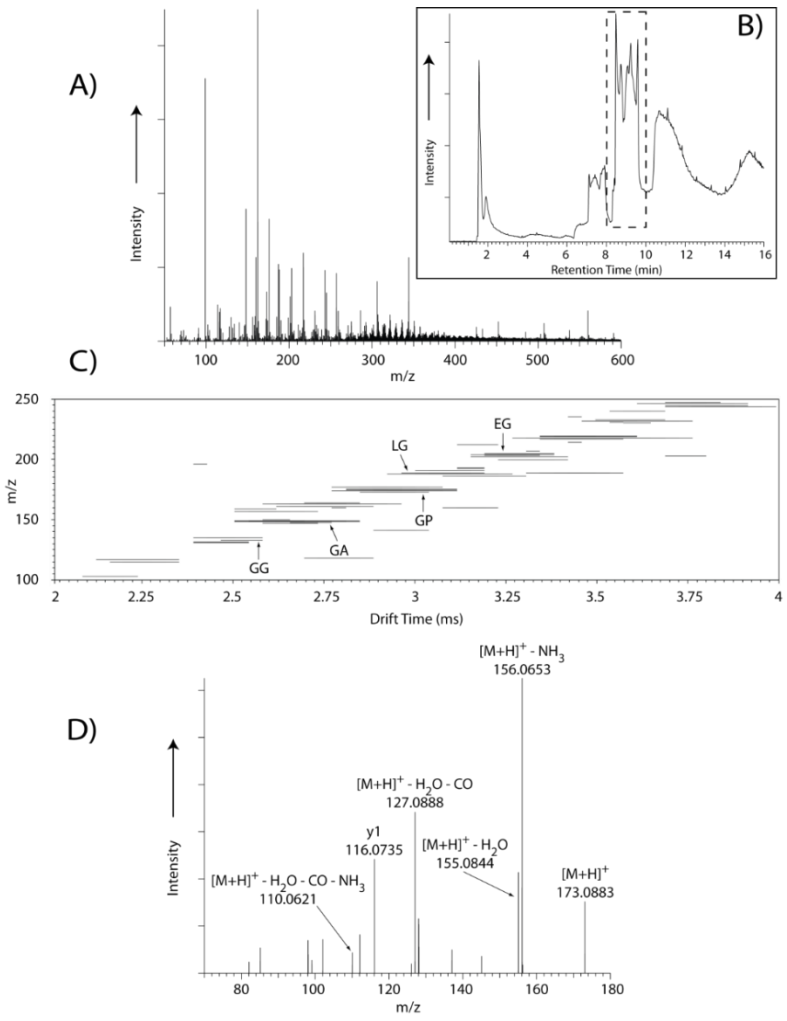

Figure 4.5. UPLC-Q-TWIMS-TOF-MS data demonstrating how dipeptides were identified in the archived cyanamide samples. Data from fraction 57-67 are shown here as an example. A) An averaged mass spectrum for the 8-10 minute region of the UPLC total ion chromatogram (B), exhibiting the vast chemical complexity of the samples studied here. C) TWIMS-TOF-MS m/z vs drift time plot for the 8-10 minute region of (B), where a suite of glycine-containing dipeptides are separated based on specific drift times. D) Following TWIMS separation, MS/MS spectra were obtained to confirm dipeptide detection by the presence of the $[M+H]^+$ ion, along with its corresponding fragment ions produced by CID in the transfer cell; shown here is the MS/MS spectrum for glycylproline. Here, GG = glycylglycine, GA = glycylalanine, GP = glycylproline, LG = leucylglycine, EG = glutamylglycine.

Table 4.4. Glycine-containing dipeptides detected in the original cyanamide samples.

Dipeptide	Theoretical $[M+H]^+/y_1$	Experimental $[M+H]^+/y_1$	$[M+H]^+/y_1$ Δm (mDa)	Molar Abundance (relative to Ala = 1)
Gly-Gly	133.0613/76.0399	133.0670/76.0468	5.7/6.9	6.96×10^{-4}
Gly-Ala	147.0770/90.0555	147.0772/90.0557	0.2/0.2	3.83×10^{-3}
Gly-Thr	177.0875/120.0661	177.0913/120.0676	3.8/1.5	5.26×10^{-4}
Gly-Pro	173.0926/116.0712	173.0883/116.0735	4.3/2.3	1.34×10^{-2}
Pro-Gly[a]	173.0926/76.0399	173.0920/-	0.6/-	2.65×10^{-3}
Gly-Val/Val-Gly	175.1083/(118.0868/76.0399)	175.1077/(118.0845/76.0353)	0.6/(2.3/4.6)	7.57×10^{-3}
Gly-Glu	205.0824/148.0610	205.0827/148.0596	0.3/1.4	2.56×10^{-3}
Glu-Gly	205.0824/76.0399	205.0829/76.0441	0.5/4.2	4.11×10^{-3}
Leu-Gly	189.1239/76.0399	189.1254/76.0416	1.5/1.7	6.78×10^{-4}
Ala-Pro[b]	187.1083	187.1084	0.1	-
Glu-Thr/Thr-Glu[b]	249.1087	249.1166	7.9	-
Glu-His[b]	285.1199	285.1179	2.0	-
Ser-Val[b]	205.1188	205.1187	0.1	-
Pro-Pro-Gly[b]	270.1454	270.1434	2.0	-
Asp-Asp-Gly[b]	306.0937	306.0980	4.3	-
Ser-Ser-Arg[b]	349.1836	349.1833	0.3	-

[a] The corresponding y_1 ion was not detected for Pro-Gly. [b] Dipeptides and tripeptides that were tentatively identified, but not quantitated.

Table 4.5. Glycine-containing DKPs detected and quantified in Miller's archived cyanamide experiment samples. MS/MS analysis of fragmentation ions was not performed for glycine-containing DKPs.

DKP	Theoretical Mass $[M+H]^+$	Experimental Mass $[M+H]^+$	$[M+H]^+$ Δm (mDa)	Molar Abundance (rel to Ala = 1)
Cyclo(Gly-Gly)	115.0508	115.0602	9.4	0.24
Cyclo(Gly-Pro)	155.0821	155.0822	0.1	0.16
Cyclo(Leu-Gly)	171.1134	171.1133	0.1	0.10

The findings of dipeptides in the archived samples were further confirmed by performing an acid hydrolysis on a portion of each sample,[34] analyzing the hydrolyzed fractions, and verifying that the peptide bonds had been cleaved, yielding their AA residues. Additionally, identical dipeptide and DKP analyses, as reported for Miller's cyanamide samples, were simultaneously carried out on ED samples from Miller's 1958 H_2S experiment,[18] which did not incorporate a condensing reagent. Peptides were not detectable in the H_2S samples, providing added evidence to suggest that the presence of a condensing reagent facilitates AA polymerization.

The ratio of AAs to dipeptides in the cyanamide samples was calculated to be approximately 1000:1-10, which agrees well with experimental data indicating that under equilibrium conditions, the AA to dipeptide ratio is approximately 1000:1.[36] Furthermore, experimental data suggest that, at equilibrium, the dipeptide to DKP ratio should be on the order of 1:10,[37] and this ratio was determined to be 1:10-20.5 in the samples studied here. The cyclic nature of the DKP is responsible for its higher thermodynamic stability, and thus, greater equilibrium abundance than the linear dipeptide.[37] However, equilibrium, alone, cannot explain the presence of dipeptides in Miller's cyanamide experiment samples because the samples from Miller's H_2S experiment,[18] which were simultaneously analyzed in an identical fashion, did not show the presence of dipeptides. The presence of dipeptides is also influenced by such factors as temperature and pH.[38]

4.4.3 *Dipeptides Produced by the Repeated Cyanamide Experiment*

In addition to investigating the archived cyanamide samples with modern analytical techniques, Miller's cyanamide experiment was repeated to generate fresh samples for further study. The analysis of the aqueous solution from the repeated

experiment was compared to that of the original samples. Upon analyzing the repeat experiment samples, there was evidence to indicate that polymerization products had been formed. Among these biomolecules were some of the same peptides that were found in the original samples, including glycylglycine (Figure 4.5.A) and 2,5-diketopiperazine (Figure 4.5.B). Some peptides not reported in the original samples were also detected in the repeated experiment samples. These included such organic molecules as alanylalanine (Figure 4.5.C) and alanine anhydride (Figure 4.5.D). These compounds were identified based on analyte retention times corresponding to developed MRM transitions for target dipeptides and DKPs. The findings of the repeated cyanamide experiment help corroborate the results obtained from analyzing the archived samples in suggesting that cyanamide can induce peptide formation under such a mimicked primitive Earth environment.

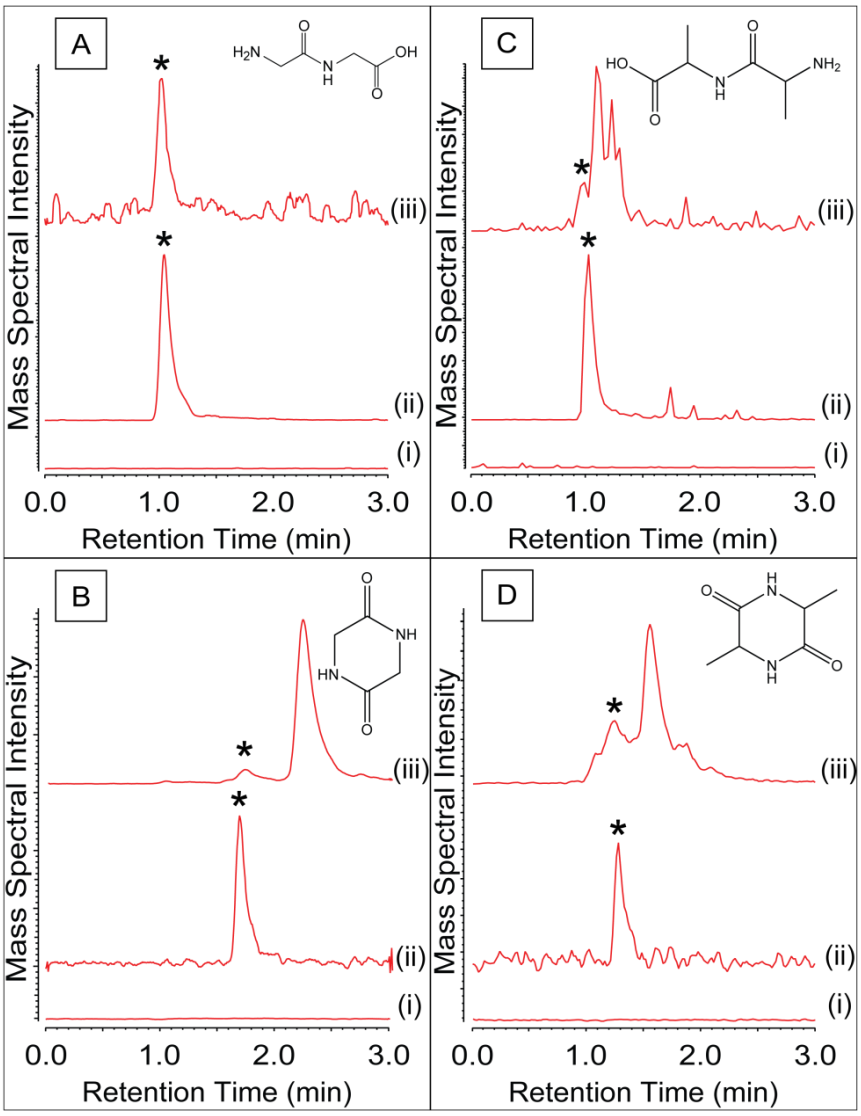

Figure 4.6. Dipeptides detected in the repeated cyanamide experiment. MRM chromatograms of the m/z 133.1 → 76.1 transition, yielding the detection of glycylglycine (A), the m/z 115.1 → 87.0 transition, demonstrating the detection of 2,5-diketopiperazine (B), the m/z 161.1 → 90.1 transition, providing the detection of alanylalanine (C), and the m/z 143.2 → 115.0 transition, facilitating the detection of alanine anhydride (D). Asterisks denote peaks that represent the analyte of interest. In all cases, trace (i) corresponds to an analytical blank, trace (ii) corresponds to a 10 µM standard of the analyte in question, and trace (iii) corresponds to a sample collected from the repeated cyanamide experiment. The sample trace in B) was from a directly analyzed portion of the duplicate cyanamide experiment aqueous phase, whereas the sample traces for A), C), and D) were from a 10x concentrated aliquot of the repeated cyanamide experiment aqueous solution. Included in the top-right corner of each quadrant is the structure of the dipeptide of interest, to provide a visual representation of the types of analytes targeted in this study.

4.4.4 *Polymerization Chemistry Observed in Heating Experiments*

Heating experiments on aqueous solutions were carried out to evaluate how dipeptide synthesis could proceed under mildly basic conditions. Solutions containing only AAs in the presence of cyanamide or its dimer, dicyandiamide (2-cyanoguanidine), were prepared at pH 1-2, pH 6-7, pH 9-10, and pH 12-13 and heated at 50°C. Although dicyandiamide was not directly introduced into the discharge apparatus, its potential as a condensing reagent was evaluated because cyanamide is known to dimerize readily in basic solutions[39] and because dicyandiamide is also a proposed prebiotic condensing reagent.[40] Analyses of the various heated pH solutions confirmed that dipeptide synthesis only took place at acidic pH values, and that dicyandiamide was a more prolific condensing reagent than cyanamide (Figure 4.6).

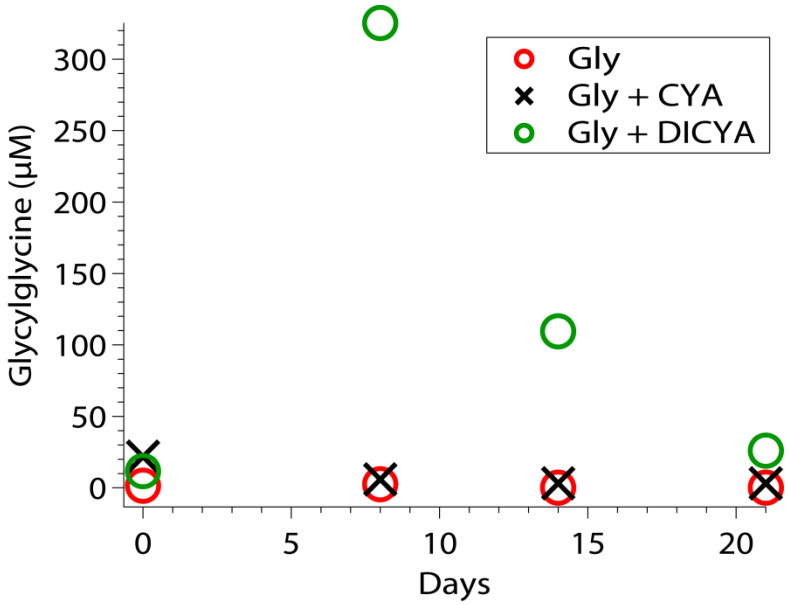

Figure 4.7. Change in glycylglycine concentration over time as observed from the analysis of heating experiment samples that contained glycine, and cyanamide or dicyandiamide in an acidic solution. Here, CYA = cyanamide, and DICYA = dicyandiamide. The pH values of the solutions shown here are as follows: Gly = 1.16, Gly + CYA = 1.20, Gly + DICYA = 1.50.

The formation of dipeptides in a mildly basic medium (pH 8-10), created by NH_3 in the spark discharge experiment, in both the original and repeated cyanamide experiments, contrasts with previous reports indicating that acidic conditions are necessary to promote cyanamide-mediated AA polymerization. As noted previously, in acid solutions with pH values less than the pK_1 of the AA,[22] the reacting AA species would be H_3N^+-C(RR′)-COO^-. Because the pK_a of the COOH group is 2-2.5, as pH increases above pH ~3 the concentration of this reactive species declines, and at the pH of the spark discharge experiments the abundance of the protonated carboxylic acid is thus expected to be negligible. This suggests that perhaps one or more components intrinsic to the spark discharge experiment may be responsible for facilitating the observed AA polymerization. Possible candidates include NH_3 , as NH_3 was included in the initial experimental conditions, and the amino acid amides and aminonitriles, both of which are Strecker synthesis intermediates.[5, 41]

Upon adding NH_3, in the form of NH_4Cl, to the pH 9-10 solutions containing glycine, glycine + cyanamide, or gylcine + dicyandiamide, the solutions were heated at 50 °C and analyzed for dipeptides. However, the presence of NH_3 resulted in negligible quantities of polymerization under basic conditions, similar to the pH of the repeated cyanamide spark discharge experiment aqueous solution, which had a pH of ~10. This result lead to the conclusion that NH_3 played a negligible role in cyanamide-mediated amino acid polymerization.

Next, the amino acid amide and aminonitrile were investigated to better understand the dipeptide synthetic route in the cyanamide spark discharge experiment and to evaluate the possible roles of these species in facilitating polymerization under

mildly basic conditions. Glycinamide and glycinonitrile were added separately to the aforementioned stock pH 9-10 solutions containing glycine, glycine + cyanamide, or glycine + dicyandiamide. After being subjected to heating at 50°C for ranges of times up to 3 weeks, these solutions were analyzed for dipeptides and DKPs. Solutions that were not heated were frozen at 0°C for use as a t = 0 control.

The analysis of the heating experiment samples containing glycinamide and glycinonitrile demonstrated that at a mildly basic pH, cyanamide and dicyandiamide reacted readily in the presence of the amino acid amide, and a factor of ~2-4 times less with the amino acid nitrile, to generate dipeptides (Figure 4.7). Over a 3 week period, upwards of ~7 µM of glycylglycine was synthesized in a basic solution that contained an AA, the corresponding amino acid amide or aminonitrile, and cyanamide or its dimer.[42]

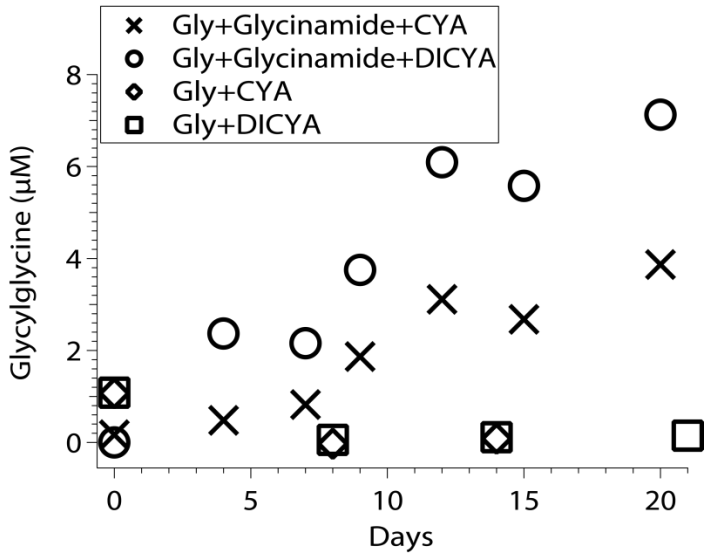

Figure 4.8. Change in glycylglycine concentration over time as observed from the analysis of heating experiment samples that contained glycine, and cyanamide or dicyandiamide, as well as glycine, glycinamide, and cyanamide or dicyandiamide, in a basic solution. Here, CYA = cyanamide, and DICYA = dicyandiamide. The pH values of the solutions shown here are as follows: Gly + CYA = 9.15, Gly + DICYA = 9.18, Gly + Glycinamide + CYA = 8.85, Gly + Glycinamide + DICYA = 8.42.

These results indicate that the presence of the amino acid amide, or amino acid nitrile, is involved in the cyanamide-mediated AA polymerization reaction. It should be noted that under these conditions dicyandiamide facilitated polymerization ~2 times greater than did cyanamide, therefore dicyandiamide performed as the superior condensing reagent under mildly basic conditions, in addition to acidic conditions. This suggests that in the ED experiment, the dimerization of cyanamide, which is fastest at pH 9.6,[39] close to the pH of the repeated cyanamide experiment, may have produced dicyandiamide within the discharge solution, where it then likely played a greater role in initiating AA polymerization, than cyanamide itself.

Figure 4.8, based in part on other work[42] and the results presented here, shows a possible mechanism for the dicyandiamide-mediated synthesis of linear peptides at pH 9-10, while highlighting the role of the amino acid amide. Here, the carbodiimide form of cyanamide dimerizes to dicyandiamide under mildly basic conditions and then can be attacked by the nucleophilic carboxylate group of the AA (e.g. glycine) to form the activated AA (I). At pH values >~8, the amino group of glycinamide (pK_a of ~8)[22] can attack the activated AA, the product of which can subsequently be hydrolyzed to ultimately give the linear dipeptide (II). Note that the pK_a of the amino group of glycinamide is lower than the pK_a of the amino group of glycine, which is ~9.8.[22] As a consequence, the $–NH_2$ of glycinamide will be less protonated under such a regime, while the $–NH_2$ of glycine will be more protonated. Thus, glycinamide is a better nucleophile than free glycine in the spark discharge pH regime. However, it is worth noting that at pH values greater than the pK_a of the amino group in glycine, the unprotonated AA would also be a reactive species. Also shown in Figure 4.8 are several possible routes by which

a second activated monomer (IA) can be formed as a by-product, which, itself, may undergo similar reactions as dicyandiamide to form the linear dipeptide. These possible additional dipeptide formation pathways may help explain why dicyandiamide induces greater quantities of AA polymerization than does cyanamide.

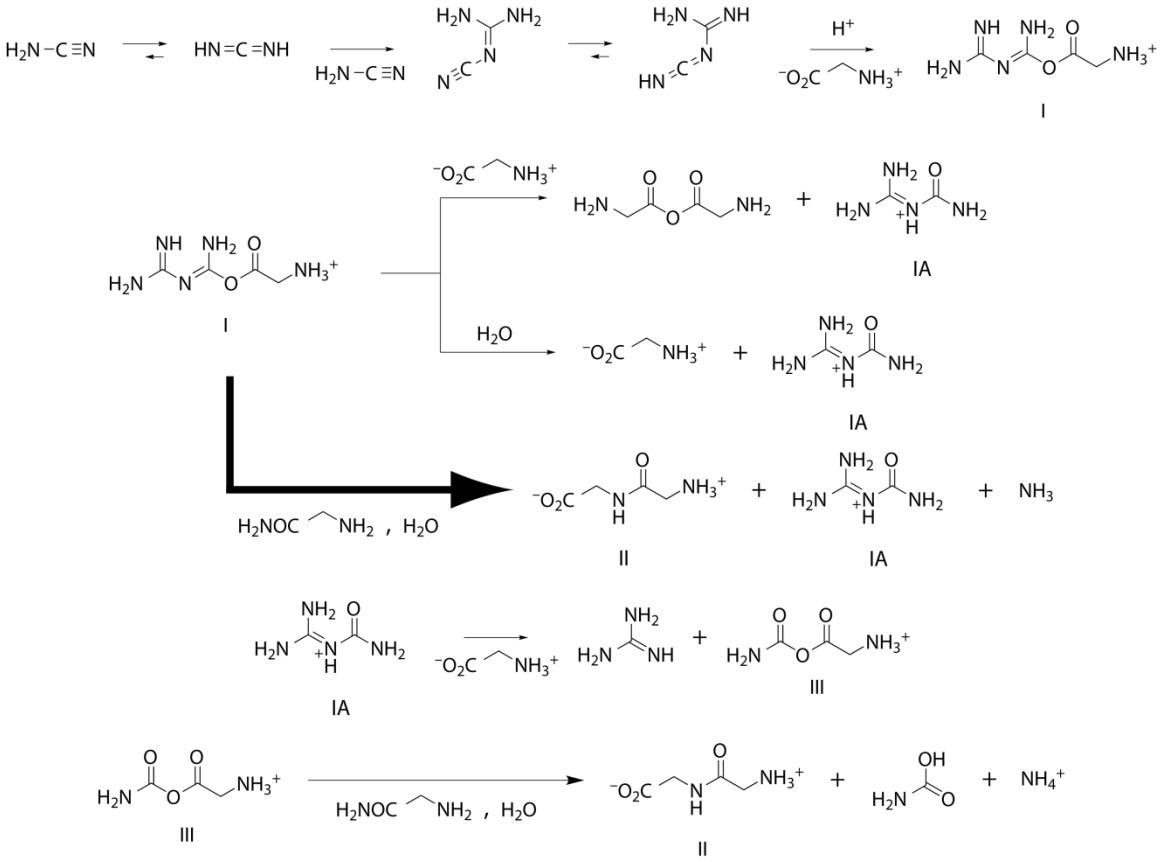

Figure 4.9. Scheme showing the dicyandiamide-mediated reactions involved in AA polymerization. The main dipeptide formation pathway is highlighted by the bolded arrow, whereby the attack of the amino acid amide on the reactive intermediate (I) first yields the peptide amide, which is then hydrolyzed[43] to give the linear dipeptide (II).

Despite the likelihood that dicyandiamide played the dominant role in facilitating polymerization, the influence of cyanamide on AA polymerization within the discharge reaction should not be neglected. A proposed mechanism for the cyanamide-mediated AA polymerization process is shown in Figure 4.9.

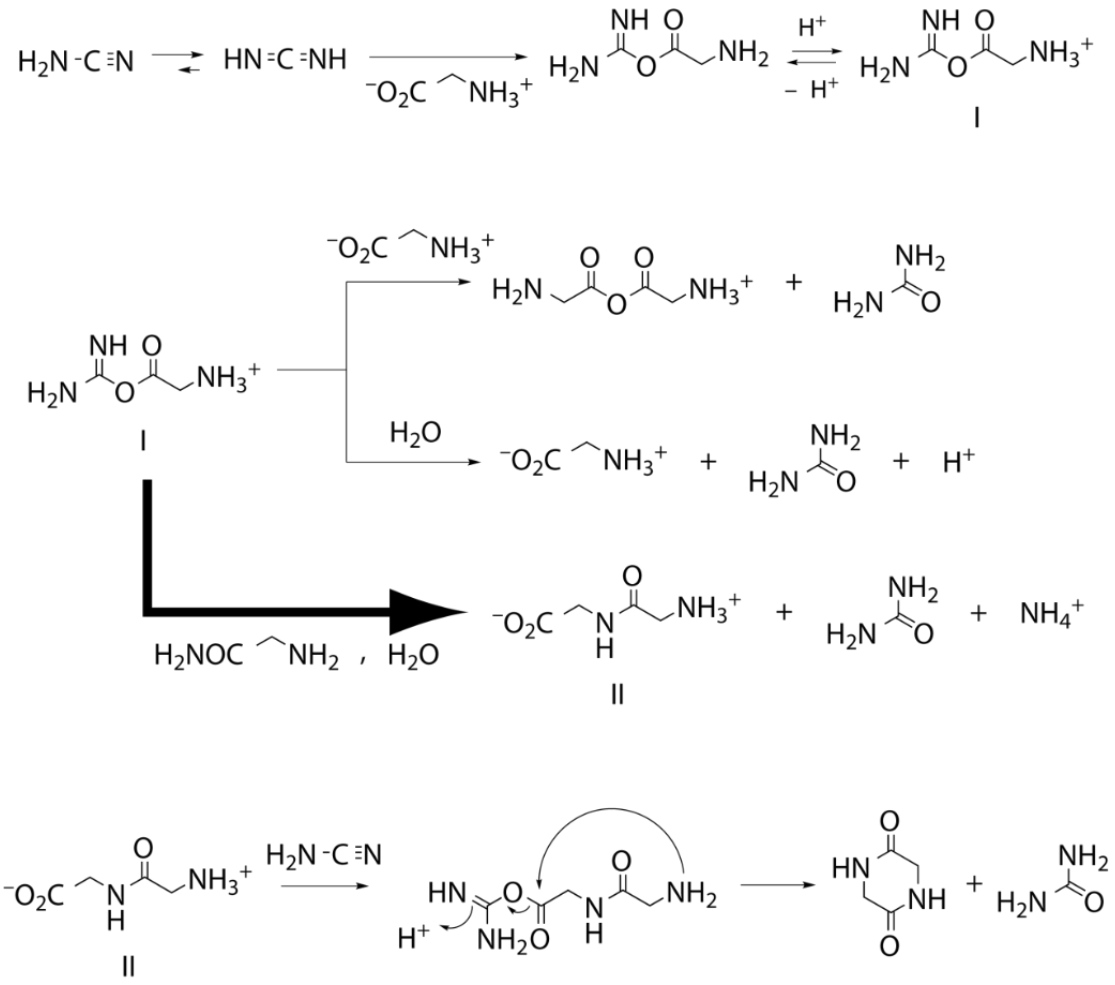

Figure 4.10. Scheme showing cyanamide-mediated AA polymerization reactions. Cyanamide reacts with an AA, glycine is shown as an example, to form an activated monomer (I). Once (I) is formed, it can undergo reaction with glycine to yield glycine anhydride, or with water to produce the original AA, among other by-products. The pK_a of the amino group in the amide, glycinamide is shown here as an example, is ~ 8 at pH values >~8,[22] therefore the reaction of (I) with the amide is the favored reaction (bolded arrow) within a basic chemical environment to form the linear dipeptide (II). Product (II) can then react directly with cyanamide to undergo ring formation to yield the DKP. The scheme portrayed here is based in part on previously reported literature.[42]

Hydrolysis of the amino acid amide to yield the AA is a potentially inhibitive pathway to dipeptide formation that should be considered. At a pH of 9.75 and a temperature of 55 °C, the half-life of glycinamide is ~ 3 days, while at a pH of 7.95 and a temperature of 75 °C, the half-life of glycinamide is ~7 days.[43] Extrapolating from these

data, and considering that the cyanamide experiment was also mildly basic and that the reaction flask was no longer heated after the introduction of cyanamide, glycinamide is expected to have had a sufficiently long lifetime to help facilitate the observed polymerization chemistry. Likewise, it is probable that the same is true in the case of the heating experiments that were performed, which mimicked the spark discharge solutions.

4.5 Conclusions

The findings detailed here demonstrate the simultaneous synthesis of both simple and complex molecules under plausible prebiotic conditions. Miller's cyanamide experiment marks the first effort to study a prebiotic condensing reagent for its implications to life's origins. Additionally, the results obtained here highlight the potential importance of condensing reagents in providing a mechanism to explain how simple organic compounds like AAs may have polymerized to form more complex molecules, such as dipeptides. From these experiments, it was determined that the amino acid amide and aminonitrile intermediates of the Strecker synthesis of amino acids played crucial roles in facilitating cyanamide-mediated amino acid polymerization under mildly basic conditions. Furthermore, the amino acid amide proved to be the more reactive intermediate to encourage peptide synthesis, particularly when compared to the AA and condensing reagent, alone. Additionally, from these findings it can be concluded that dicyandiamide is a better condensing reagent than cyanamide. The synthesis of dipeptides and DKPs by the cyanamide polymerization reaction may have additional implications, as some dipeptides and DKPs have been found to have catalytic properties that may have been important on the primordial Earth.[44-46]

4.6 Acknowledgements

This work is dedicated to Stanley L. Miller, whose seminal research in the early 1950s brought legitimacy to the origins of life field and gave rise to the field of prebiotic chemistry. The work in this chapter is also dedicated to Joan Oró, who, like Miller, conducted electric discharge experiments to study a variety of possible abiotic chemistries, but also investigated cyanamide as a plausible prebiotic condensing reagent.

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CHAPTER 5: QUANTITATION OF α-HYDROXY ACIDS IN COMPLEX PREBIOTIC MIXTURES VIA LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

Adapted from:
Parker, E. T.; Cleaves, H. J.; Bada, J. L.; Fernández, F. M.(2016) Quantitation of α-Hydroxy Acids in Complex Prebiotic Mixtures via Liquid Chromatography-Mass Spectrometry. *Rapid Communications in Mass Spectrometry*, in press.

5.1 Abstract

Spark discharge experiments, like those performed by Stanley Miller in the 1950s, generate complex mixtures that are analytically challenging and are known to contain the monomeric building blocks of biopolymers, including α-AAs. A major uncertainty in the origins of life field is how monomer species could have formed primitive polymers. It was recently shown that when subjected to environmental cycling, a mixture of α-AAs and α-hydroxy acids (AHAs) can polymerize, yielding simple depsipeptides (peptides with both amide and ester linkages). To better understand this chemistry, it is imperative to constrain which AHAs may be synthesized under possible primordial environments and at what concentrations. However, only sparse reports exist in the literature demonstrating the synthesis of AHAs from prebiotic simulations. Also, reported analytical techniques for AHAs suffer from such drawbacks as the need for derivatization, long analysis time, or limited detection capabilities. In this chapter, a fast and direct (without derivatization) analysis method for AHAs is reported using UPLC/QqQ-MS. Combining ion-pairing chromatography separation and MRM detection allowed for the rapid analysis of 10 AHAs in a single chromatographic run. This method was used to analyze the products of MU-type ED experiments, including the repeated

cyanamide spark discharge experiment. Under basic conditions, akin to those produced by typical ED experiments, AHA synthesis tends to be disfavored. However, it was found that AHAs, such as glycolic acid, lactic acid, malic acid, and α-hydroxyglutaric acid were detected in the low micromolar concentration range. This work demonstrates that these species, at their respective concentrations, warrant further investigation for their roles in possible prebiotic depsipeptide synthesis.

5.2 Introduction

Previous analyses of ED experiments have focused on the detection and quantitation of biopolymer building blocks such as α-AAs[1] and to a much lesser extent, α-hydroxy acids (AHAs).[2] The formation of peptides[3] from the polymerization of prebiotic monomers, however, continues to be a focus of intense research. Abiotic peptide synthesis is often inhibited by the formation of DKPs,[4-6] whose high stability can reduce the polymerization of AAs beyond simple dimers. Furthermore, the activation energy barrier to the polymerization of α-AAs gives rise to the problem that with conditions such as elevated temperatures where polymerization becomes more favorable, oligopeptide synthesis often results in significant degradation of the starting materials.[7]

Recently, oligomerization of malic acid has been achieved through simulated dry/wet environmental cycling.[8] Such environmental cycling has also been shown to form mixed amide-ester linkages from a combination of AHAs and AAs, which gradually become amide-enriched depsipeptides with up to n = 10 units.[9] Depsipeptide ester bonds are less stable to hydrolysis than amide bonds,[8] and consequently facilitate rapid sequence evolution and primitive peptide synthesis. Detecting AHAs in ED experiments is therefore of essence to evaluate the abundance and diversity of building blocks

available to form depsipeptides. Figure 5.1 illustrates the general structure of depsipeptides, and highlights the ester and amide linkages, as contributed from AHAs and α-AAs, respectively. To this end, a method for detecting and accurately quantifying AHAs in model prebiotic reaction mixtures is needed.

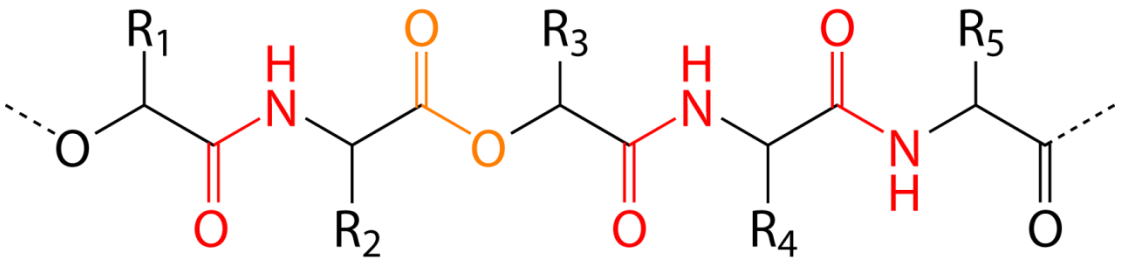


Figure 5.1. General depsipeptide structure composed of both amide linkages from α-AAs (red) and ester linkages from AHAs (orange).

Hydroxy acids have numerous vital implications for a variety of fields of study, including food science,[10] biomedical materials,[11] metabolic disease screening,[12] and meteorite studies,[13] thus justifying the need for a robust analytical platform for these analytes beyond the needs of prebiotic chemistry. However, previously reported analytical methods suffer from innate shortcomings, such as the use of time consuming GC-MS,[14] the need for complex derivatization procedures with expensive reagents,[15] or in some cases, using derivatization reagents that are potentially explosive.[16] Furthermore, more rapid approaches reported in the past have only demonstrated the detection of a limited number (1 to 5) of AHAs,[10, 17-19] or have not used mass spectrometric detection.[20]

Ideally, a new AHA analytical method should be able to detect and quantify a larger suite of AHAs within a single chromatographic run without derivatization. Due to their relatively low molecular weight and polar nature, however, separation of AHAs is inherently challenging with standard reversed phase liquid chromatographic approaches.

Therefore, this chapter reports a new method for the rapid analysis of 10 underivatized AHAs using UPLC/QqQ-MS, where the chromatographic separation is enabled using hexylamine as an ion-pairing agent. This method is then applied for the first time to complex mixtures produced by four MU-type ED experiments simulating possible primordial Earth conditions.

5.3 Materials and Methods

5.3.1 *Chemicals and Reagents*

Water used for AHA studies was Nanopure Diamond quality (18.2 MΩ cm), while water used for AA studies was Millipore water (18.2 MΩ cm, <3 ppb total organic carbon). All glassware and tools used to handle samples were thoroughly washed with water, then wrapped in aluminum foil, and heated at 500 °C, overnight, in air. Unless otherwise stated, all reagents used in this study were purchased from Sigma-Aldrich, Acros Organics, Chem-Impex International, MP Biomedicals, or Fluka, with purities of 97 % to ≥99 %. Stock AHA standard solutions (10^{-4} M) were prepared by dissolving individual AHA crystals in a solution of 5 mM hexylamine in water (hexylamine manufacturer/purity: Sigma-Aldrich/99 %). The individual AHA standard solutions were subsequently mixed for their combined analysis.

Two eluents were used for UPLC/QqQ-MS analysis: (A) 5 mM hexylamine in water and (B) 90:10 methanol:10 mM NH_4OAc in water. Reagent (A) was prepared by dissolving hexylamine in water. Reagent (B) was prepared by making a 10 mM NH_4OAc solution in water, and adjusting the pH to 8.5 using 7 N NH_3 in methanol, prior to mixing with LC-MS grade methanol.

Gases used for ED experiments included ultra high purity CH_4, ultra high purity N_2, anhydrous NH_3, and ultra high purity argon (Ar). Gases were obtained from Airgas. Purities of gases were 99.99 % for CH_4, NH_3, and Ar, and 99.999 % for N_2.

5.3.2 *ED Experiment #1*

Miller collected his original cyanamide spark discharge experiment samples from a cation exchange column in fractions that corresponded to regions in which AAs and peptides were most likely to have been present, preventing the analysis of Miller's original samples for AHAs. We therefore could not analyze the original samples for AHAs, instead the repeated cyanamide experiment was analyzed. Full details regarding how the cyanamide experiment was reproduced have been reported in Chapter 4 of this thesis and in the literature.[3] However, a brief overview will be provided here. Into a 3 L reaction flask with 2 tungsten electrodes was introduced 250 mL H_2O, 300 mmHg CH_4, and 250 mmHg NH_3. The experiment was paused at 3 time points, matching the time sequence originally used by Miller, to introduce cyanamide into the reaction flask at concentrations equal to those used by Miller. Ar gas was used to help introduce the external cyanamide solution into the sealed reaction vessel.

5.3.3 *ED Experiment #2*

A gas mixture of 200 mmHg N_2/100 mmHg CH_4 over a 100 mL aqueous solution of 0.225 M $NaHCO_3$, adjusted to pH 8.7, was sparked. Sodium bicarbonate was selected as a buffering agent because the composition of prebiotic seawater has been hypothesized to be more carbonic than the modern ocean,[21-22] with upwards of ~10x more bicarbonate than today,[21] and large quantities of sodium ion,[21, 23] contributing to an abundance of sodium bicarbonate.[24] The reaction was subjected to ED for 99 hours, in 1 hour on/1 hour

off intervals. After stopping the reaction, samples were collected and stored frozen until analysis. Full details regarding gas introduction into the 3 L reaction flask, execution of a MU-type spark discharge experiment, and collection of samples for analysis following the completion of the experiment, are based on the protocol outlined in Chapter 2 of this thesis and in the literature.[25]

5.3.4 *ED Experiment #3*

An ED experiment based on previous work[26] was conducted with a N_2/CH_4 atmosphere and a 0.225 M NH_3/NH_4Cl buffered aqueous phase. The aqueous phase was chosen to be buffered by NH_3/NH_4Cl because although the concentration of NH_3 in the primitive atmosphere was likely $\leq 10^{-5}$,[27-28] this concentration of gaseous NH_3 would have contributed to relatively high concentrations of $NH_3$and $[NH_4]^+$ in seawater.[26] Additionally, chloride ion is thought to have been another dominant ion in the prebiotic ocean,[23] which, in combination with dissolved NH_3, could have contributed to the formation of NH_4Cl in the early ocean. The presence of NH_3, in the form of NH_4Cl, could have served two important functions: 1) facilitating prebiotic organic synthesis, and 2) acting as a buffer to stabilize oceanic pH. A 100 mL solution of 0.05 M ammonium chloride and 105 mmHg anhydrous NH_3 (to make the solution 17.5 mM in $\sum NH_3$ (NH_3 + NH_4^+), with a final pH of 8.7) was introduced into a 3 L flask fitted with two tungsten electrodes. Methane was introduced into the flask to a partial pressure of 110 mmHg and N_2 was added to a partial pressure of 300 mmHg. An ED was imparted across the tips of the electrodes, in a 1 hour on/off cycle, over a 96 hour time period.

5.3.5 *ED Experiment #4*

A final ED experiment was carried out, identical to ED Experiment #3, except for the use of a 200 mmHg N_2/200 mmHg CH_4 gas mixture, and the introduction of 3.708 g of synthetic schreibersite ($(Fe, Ni)_3P$)[29-30] into the liquid reservoir. The inclusion of (Fe, $Ni)_3P$ allowed for the investigation of the impact of mineral surfaces.

5.3.6 *ED Sample Analysis*

Samples generated from these ED experiments are very complex (Figure 5.1). Therefore, a targeted analytical approach is preferred for the detection and quantitation of a specific set of analytes. Samples were analyzed for AHAs by UPLC/QqQ-MS, either via direct analysis of the raw discharge solution, or by first drying the sample and then reconstituting it in a 50:50 (v:v) mixture of LC-MS grade acetonitrile and 10 mM NH_4Fo in water. The sample supernatant was then subsequently separated for analysis.

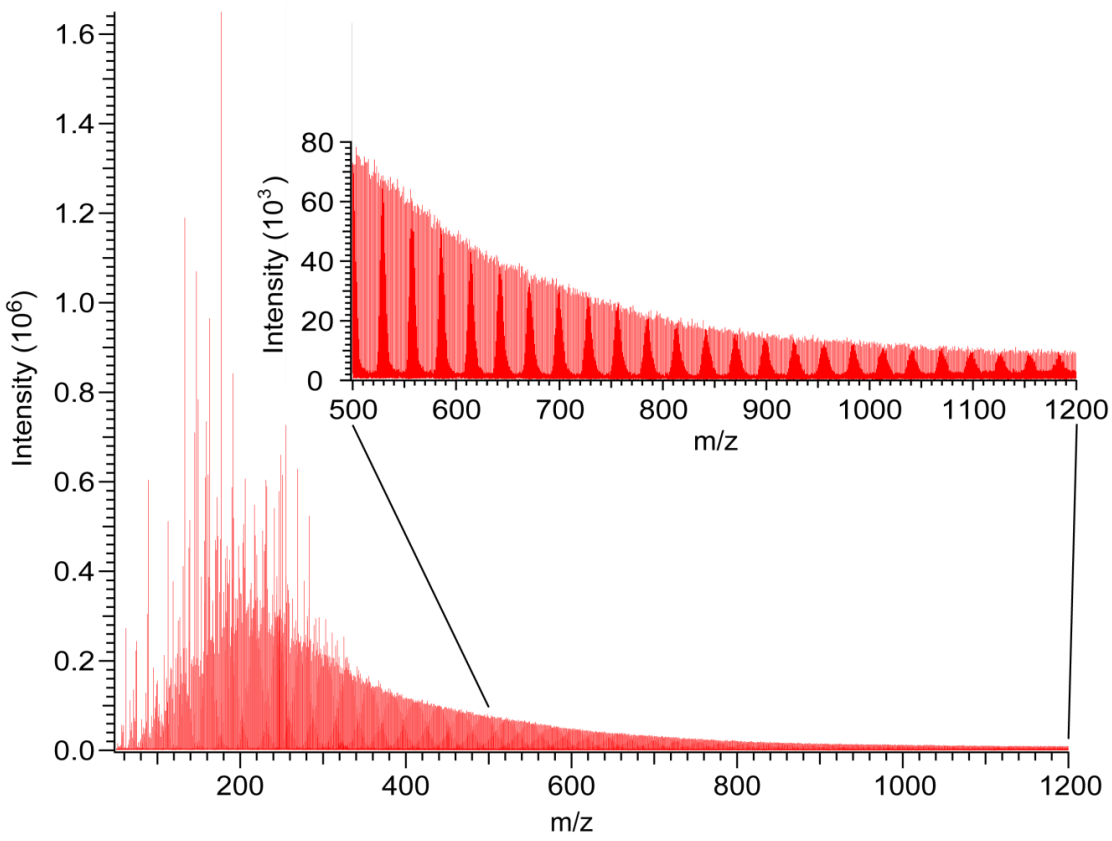


Figure 5.2. A high resolution total mass spectrum of the sample solution originating from ED Experiment #1 (including cyanamide as a condensing reagent), demonstrating the type of chemical complexity these ED experiments can generate; thousands of compounds with masses upwards of 1200 Da. These types of complex samples necessitate the use of a targeted analytical approach for the detection of AHAs. The inset is a zoomed-in look at the m/z 500 - 1200 range to illustrate the structure of the mass spectrum in the higher mass range. Note: the inset appears to possess a repeating signal unit of ~30 Da. Upon examination of progressively more zoomed in mass spectra from the same sample, it appears this trend is a visual effect produced by saturation of the display resolution. Such an effect is commonly observed when plotting very crowded mass spectra from petroleum samples, for instance. However, it should be emphasized that the possibility cyanamide may mediate the production of periodical signals of ~30 Da (as a result of the addition of a CH_2O functionality, for example) deserves further investigation, although such a study is beyond the scope of the present work.

UPLC/QqQ-MS experiments were conducted using an Agilent 1290 Infinity UPLC unit, coupled to an Agilent 6430 QqQ-MS. Mass spectral detection of target analytes was achieved by operating the mass spectrometer in MRM mode.

Chromatography was performed using a 2.1 x 100 mm, 1.7 µm particle size Waters

ACQUITY UPLC BEH C18 column. The hexylamine ion-pairing chromatographic conditions employed were based on work reported elsewhere,[31] but were specifically modified for this application. The mobile phases used were (A) 5 mM hexylamine in water and (B) 90:10 methanol:10 mM NH_4OAc in water. Target AHAs were eluted using the following gradient: 0-2 min, 0 % B, 2-6 min, 0-20 % B, 6-10 min, 20 %B, 10-11 min, 20-0 % B, 11-15 min, 0 % B. The buffer flow rate used was 0.25 mL min^{-1}. The column was kept at 50 °C. The autosampler tray was maintained at a temperature of 5 °C. Sample runs used an injection volume of 10 µL. The QqQ-MS utilized an ESI source operated in negative ion mode. Ion source conditions included a capillary voltage of 3 kV, a nebulizing gas (N_2) pressure of 15 psi, and a desolvation gas (N_2) flow rate of 6 L min^{-1} and temperature of 300 °C.

Target AHAs were glycolic acid (2-hydroxyacetic acid), lactic acid (2-hydroxypropanoic acid), α-hydroxybutyric acid (2-hydroxybutanoic acid), α-hydroxyisobutyric acid (2-hydroxy-2-methylpropanoic acid), α-hydroxy-α-methylbutyric acid (2-hydroxy-2-methylbutanoic acid), α-hydroxyisovaleric acid (2-hydroxy-3-methylbutanoic acid), α-hydroxycaproic acid (2-hydroxyhexanoic acid), α-hydroxyisocaproic acid (2-hydroxy-4-methylpentanoic acid), malic acid (2-hydroxybutanedioic acid), and α-hydroxyglutaric acid (2-hydroxypentanedioic acid).

ED samples were prepared for AA analysis by first drying down a 100 µL aliquot of the aqueous layer under vacuum in an open test tube. These samples were then sealed inside larger test tubes containing 1 mL of doubly distilled 6 N HCl. Further details regarding the acid-vapor hydrolysis protocol implemented can be found elsewhere.[32] The outer tubes were opened and the inner tubes containing the samples were carefully

removed. 1 mL of Millipore water was then added to the dried, hydrolyzed sample and this was vortexed and centrifuged briefly on a benchtop centrifuge to pellet insoluble material. 200 µL of this solution were desalted by using cation-exchange resin (BIO-RAD AG50W-X8, 100–200 mesh, H^+ form), and the AAs were recovered via elution with 2 M NH_4OH (prepared by dissolving gaseous NH_3 in Millipore water). 5 µL of 10^{-4} M DL-4-fluorovaline was added to each sample as an internal standard to estimate amino acid recoveries from desalting and OPA/NAC derivatization. Additional considerations regarding sample desalting are provided in the literature.[33]

After desalting, the AAs in the NH_4OH eluates were dried under vacuum at 30° C to remove excess NH_3. The residues were then re-dissolved in 100 µL of Millipore water. Prior to analysis, 10 µL of each sample solution were mixed with 10 µL of 0.1 M sodium borate and 5 µL of OPA/NAC, and then derivatized for 1 minute, as described previously.[34] The derivatization reaction was quenched with 75 µL of 0.1 M aqueous hydrazine hydrate, the preparation of which is described by Glavin et al.[35] Samples were then immediately analyzed by UPLC-FD/TOF-MS. A Waters ACQUITY UPLC, Waters ACQUITY fluorescence detector, and a Waters LCT Premier TOF-MS equipped with an ESI source operated in positive ion mode, were used. Details of the TOF-MS settings and the amino acid quantification methods used for these analyses are described elsewhere.[36] The UPLC-FD/TOF-MS technique has a detection limit in the low femtomole range for these amino acid derivatives. Products were identified based on three criteria: 1) they must be amine-functional group containing molecules to produce fluorescence signals using the given emission wavelength, 2) the correspondence of their chromatographic retention time with that of a known standard, and 3) the accurate mass of their $[M+H]^+$

ions. There is a slight time offset between the FD and MS trace signals due to the tandem detector arrangement.

5.3.7 *Data Analysis*

Calibration curves were constructed by analyzing AHA standard solutions in triplicate. Target analytes were prepared at 10 concentrations: 0.1 µM, 0.5 µM, 1.0 µM, 5.0 µM, 10.0 µM, 20.0 µM, 40.0 µM, 50.0 µM, 60.0 µM, and 80.0 µM.

Linear regression analysis was applied by fitting analyte peak areas vs. respective analyte concentrations. For each analyte, peak area standard deviations generally increased with an increase in concentration. As a result, a weighted linear regression analysis was performed to account for the heteroscedasticity of the acquired calibration data.[37-38] The weights (w_i) that were applied are defined as:

$$\boldsymbol{w_i = \frac{s_i^{-2}}{\sum_i^n s_i^{-2}/n}} \tag{4.1}$$

where n is the number of standard concentrations used to generate the calibration curve, and s_i is the standard deviation of the peak area measurements made across standard triplicates. These weights were then implemented to calculate the coordinates of the weighted centroid ($\bar{x}_w, \bar{y}_w$):

$$\boldsymbol{\bar{x}_w = \frac{\sum_i^n w_i x_i}{n}} \tag{4.2}$$

$$\boldsymbol{\bar{y}_w = \frac{\sum_i^n w_i y_i}{n}} \tag{4.3}$$

where x_i is the concentration of the analyte and y_i is the measured peak area corresponding to x_i. The coordinates of the weighted centroid were then used to determine the weighted slope:

$$\boldsymbol{b_w = \frac{\sum_i^n w_i x_i y_i - n\bar{x}_w \bar{y}_w}{\sum_i^n w_i x_i^2 - n\bar{x}_w^2}} \tag{4.4}$$

and the weighted y-intercept:

$$\boldsymbol{a_w = \bar{y}_w - b_w \bar{x}_w} \tag{4.5}$$

Based on the algebraic form of the equation for a weighted straight line calibration plot:

$$\boldsymbol{y_i = a_w + b_w x_i} \tag{4.6}$$

the $\hat{y}_i$ component of the y-residuals $(y_i - \hat{y}_i)$ can be calculated as:

$$\boldsymbol{\hat{y}_i = a_w + b_w x_i} \tag{4.7}$$

after inserting the known concentration of the standard analyte, and the calculated values for the weighted slope and weighted y-intercept, all into equation (4.7). The weighted, random error in the y-direction ($s_{(y/x)w}$) was then calculated using:

$$\boldsymbol{s_{(y/x)w} = \sqrt{\frac{\sum_i^n w_i (y_i - \hat{y}_i)^2}{n-2}}} \tag{4.8}$$

Lastly, the LOD and limit of quantitation (LOQ) were calculated as $3s_{(y/x)w}$ and $10s_{(y/x)w}$, respectively. These products provide a signal intensity-based estimation of the LOD and LOQ, which are then plugged into equation (4.6) for y_i, where, when using the previously calculated values for weighted slope and weighted y-intercept, the resultant molar concentrations associated with the LOD and LOQ can be obtained as x_i.

Uncertainty estimates (δ_x) of standard analyte measurements were approximated using standard deviations of three separate measurements ($n = 3$), yielding a standard error of:

$$\boldsymbol{\delta_x = s_i (n-1)^{-1/2}} \tag{4.9}$$

Confidence intervals were calculated by performing a two-tailed t-test. First, the weighted standard deviations for the slope, s_{b_w}, were calculated as:

$$s_{b_w} = \left[\frac{n\left[s_{(y/x)w}\right]^2}{(n[\sum_i^n w_i x_i^2] - [\sum_i^n w_i x_i]^2)}\right]^{1/2} \quad (4.10)$$

Once the value of s_{b_w} is obtained, the confidence interval is then calculated by multiplying s_{b_w} by the tabulated t-value at the 95% confidence level (α= 0.05) with n-2 degrees of freedom. Finally, the standard deviation of the concentration estimate for an unknown analyte is:

$$s_{x_{0w}} = \frac{s_{(y/x)w}}{b_w}\left[\frac{1}{w_0} + \frac{1}{n} + \frac{(y_0 - \bar{y}_w)^2}{b_w^2(\sum_i^n w_i x_i^2 - \sum_i^n n\bar{x}_w^2)}\right]^{1/2} \quad (4.11)$$

where y_0 is the unknown analyte average signal intensity and w_0 is the weight of the average unknown analyte signal intensity. Here, w_0 equals $1/s_0^2$, where s_0 is the standard deviation of the unknown analyte measurements.

5.4 Results and Discussion

Table 5.1 and Figure 5.2 depict MRM UPLC/QqQ-MS settings and typical results for AHA standards, respectively. As seen in Figure 5.2, some analytes sharing precursor ions yield identical product ions, depending upon the fragmentor and collision cell voltages used. This results in multiple chromatographic signals appearing in a single mass chromatogram. For example, α-hydroxyisobutyric acid and α-hydroxybutyric acid share the transition m/z 103.04 → m/z 57.1. Consequently, a signal for α-hydroxyisobutyric acid was seen as peak 4 at 4.62 minutes (Figure 5.2), in addition to the α-hydroxybutyric acid signal at 4.98 minutes. Likewise, both α-hydroxy-α-methylbutyric acid, and α-hydroxyisovaleric acid were detectable at the m/z 117.05 → m/z 70.9, and

m/z 117.05 → m/z 45.0 transitions. Therefore, α-hydroxyisovaleric acid is detected at 7.17 minutes and α-hydroxy-α-methylbutyric acid at 6.24 minutes in trace 5 (Figure 5.2). Likewise, α-hydroxy-α-methylbutyric acid is detected at 6.24 minutes and α-hydroxyisovaleric acid at 7.17 minutes in trace 8 (Figure 5.2). Lastly, trace 10 contains a small α-hydroxyisocaproic acid signal appearing at 10.01 minutes, just before the α-hydroxycaproic acid signal at 10.50 minutes, as both species were detectable at the m/z 131.07 → m/z 82.9 transition (Figure 5.2).

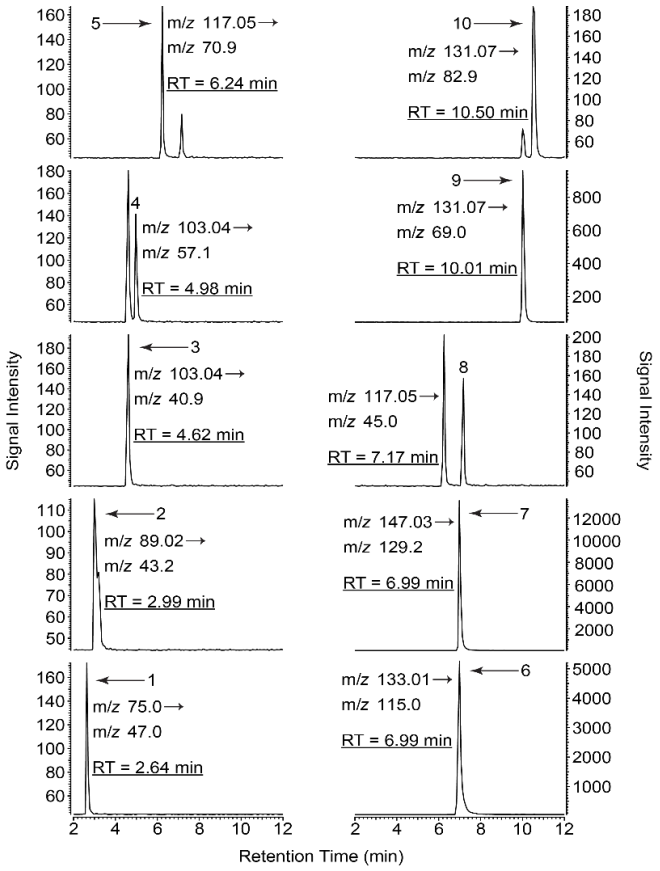
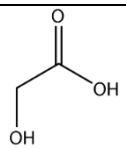
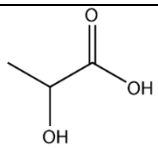
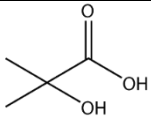
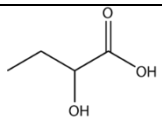
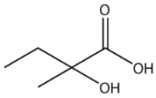
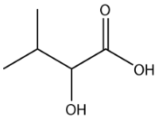
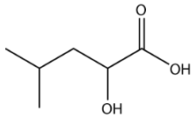
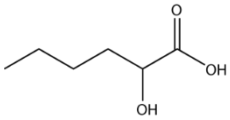
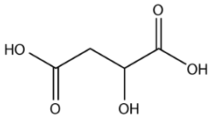
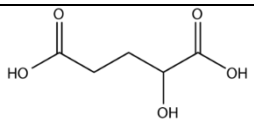


Figure 5.3. Individual extracted MRM mass chromatograms for target analytes in a mixed AHA standard, with accompanying transitions and retention times of signals of interest displayed. Signal assignments: 1 = glycolic acid, 2 = lactic acid, 3 = α-hydroxyisobutyric acid, 4 = α-hydroxybutyric acid, 5 = α-hydroxy-α-methylbutyric acid, 6 = malic acid, 7 = α-hydroxyglutaric acid, 8 = α-hydroxyisovaleric acid, 9 = α-hydroxyisocaproic acid, 10 = α-hydroxycaproic acid.

Table 5.1. Optimized MRM parameters used for AHA analysis in this study.

AHA	Structure	Precursor Ion (m/z)	Fragmentor (V)	Collision Cell (V)	Product Ion (m/z)
Glycolic Acid		75.0	64	0	47.0[Q]
Lactic Acid		89.02	64	32	43.2[Q]
α-Hydroxy-isobutyric Acid		103.04	64	20	40.9[C]
		103.04	64	28	57.1[Q]
α-Hydroxy-butyric Acid		103.04	64	28	57.1[Q]
α-Hydroxy-α-Methylbutyric Acid		117.05	64	8	45.0[Q]
		117.05	64	28	70.9[C]
α-Hydroxy-isovaleric Acid		117.05	64	8	45.0[Q]
		117.05	64	28	71.2[C]
α-Hydroxy-isocaproic Acid		131.07	26	16	69.0[Q]
		131.07	26	28	85.0[C]
		131.07	26	0	113.2[C]
α-Hydroxy-caproic Acid		131.07	64	8	45.2[C]
		131.07	64	12	73.2[C]
		131.07	64	16	82.9[Q]
		131.07	64	28	85.0[C]
Malic Acid		133.01	82	12	71.1[Q]
		133.01	82	8	115.0[C]
α-Hydroxy-glutaric Acid		147.03	64	8	129.2[Q]

[Q]Product ion monitored for use in analyte quantitation.
[C]Product ion monitored for the purpose of confirmation of analyte detection.

5.4.1 *Analytical Figures of Merit*

The performance of the method developed was evaluated by assessing the analytical figures of merit (Table 5.2). The linear range of a given transition spanned two orders of magnitude in most cases. As expected, the species with the smallest linear ranges also had the greatest sensitivity. Malic acid had transitions developed for it in which the sensitivities were near, or exceeded, 1000 μM^{-1}. Outside of α-hydroxyglutaric acid, these sensitivities were in excess of 15 times greater than the transition with the next largest sensitivity (the m/z 131.07 → m/z 69.0 transition for α-hydroxyisocaproic acid). The heightened instrumental response to malic acid caused bending of the calibration curve above 5 μM, for the quantifying transition of m/z 133.01 → m/z 71.1.

The LODs for all analytes were in the nM range. Among the lowest LODs achieved were those for α-hydroxydicarboxylic acids, which were also the species with the largest masses. This may partly be the result of a greater efficiency of ion generation due to their double carboxylic acid moieties that increase their probability of deprotonation, or because of the greater ease with which the mass spectrometer can detect larger molecules The lowest LOD recorded was that for the m/z 133.01 → m/z 71.1 transition of malic acid, which was 0.12 μM. Based on the quantifying transitions monitored for each species, all analytes had LOQ values above 1 μM, except malic acid, which had an LOQ of 0.45 μM. The high instrumental sensitivity to malic acid is largely responsible for the relatively low LODs and LOQs observed for this AHA.

Representative calibration curves for each monitored transition are shown in Figure 5.3. Each calibration curve demonstrated very strong correlation ($R^2 \geq 0.99$) between analyte peak area and standard concentration over the respective transition.

Table 5.2. Analytical figures of merit for AHA quantitation.

Analyte	Transition[a]	Sensitivity (S, µM-1)[b]	Linear Range (µM)[c]	LOD (µM)	LOQ (µM)
Glycolic Acid	75.0 → 47.0	8.36 ± 0.14	0.20 – 80.00	0.20	1.09
Lactic Acid	89.02 → 43.2	11.29 ± 0.22	0.43 – 80.00	0.43	2.38
α-Hydroxyisobutyric Acid	103.04 → 40.9	25.25 ± 0.46	0.55 – 80.00	0.55	1.87
	103.04 → 57.1	20.18 ± 1.04	0.30 – 80.00	0.30	1.52
α-Hydroxybutyric Acid	103.04 → 57.1	15.10 ± 0.93	0.94 – 80.00	0.94	2.96
α-Hydroxy-α-Methylbutyric Acid	117.05 → 45.0	22.22 ± 0.71	0.62 – 80.00	0.62	1.97
	117.05 → 70.9	15.37 ± 0.69	2.09 – 80.00	2.09	7.87
α-Hydroxyisovaleric Acid	117.05 → 45.0	15.08 ± 0.37	0.49 – 80.00	0.49	1.94
	117.05 → 71.2	5.28 ± 0.19	0.64 – 80.00	0.64	3.00
α-Hydroxyisocaproic Acid	131.07 → 69.0	56.92 ± 0.83	0.49 – 80.00	0.49	2.37
	131.07 → 85.0	5.71 ± 0.53	1.27 – 80.00	1.27	4.92
	131.07 → 113.2	4.80 ± 0.38	4.13 – 80.00	4.13	21.04
α-Hydroxycaproic Acid	131.07 → 45.2	13.52 ± 0.49	0.49 – 80.00	0.49	3.98
	131.07 → 73.2	0.72 ± 0.07	3.26 – 80.00	3.26	19.54
	131.07 → 82.9	13.94 ± 0.44	0.22 – 80.00	0.22	5.15
	131.07 → 85.0	6.24 ± 0.17	0.28 – 80.00	0.28	3.21
Malic Acid	133.01 → 71.1	989.97 ± 42.79	0.12 – 5.00	0.12	0.45
	133.01 → 115.0	2607.11 ± 273.88	0.23 – 10.00	0.23	0.81
α-Hydroxyglutaric Acid	147.03 → 129.2	1369.55 ± 13.28	0.52 – 80.00	0.52	1.95

[a]All correlation coefficients of the weighted linear regression curves were ≥ 0.99 over the respective linear ranges.
[b]Slope of the weighted linear regression (averaged peak area vs AHA concentration).
[c]Concentrations higher than 80 µM were not tested.

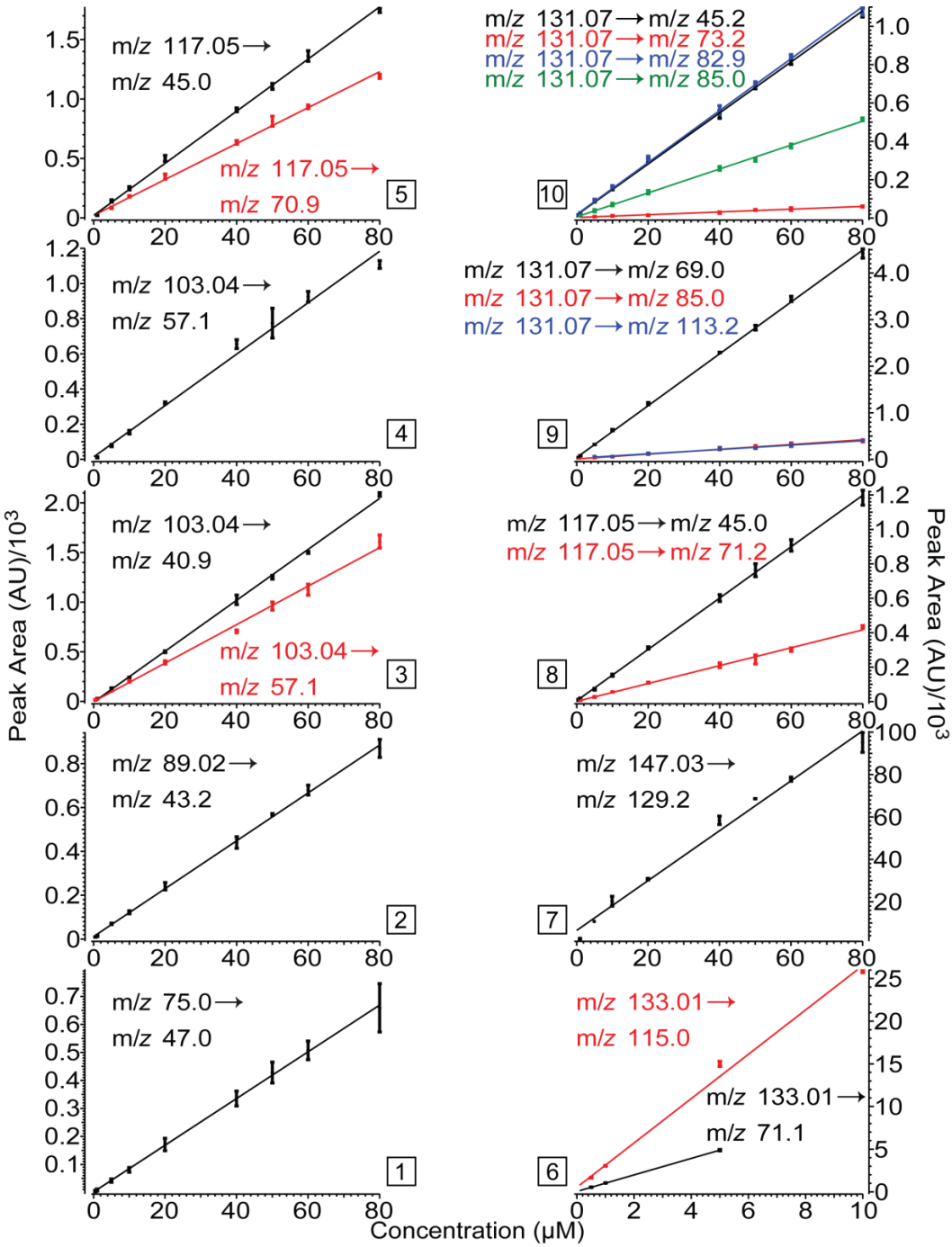


Figure 5.4. Calibration curves for each target analyte and its respective transitions. Data points included in calibration curves are only of those standard concentrations that fall within the linear range of each transition evaluated. All measurements were performed in triplicate. Error bars represent uncertainty estimates as calculated in equation (9). Analyte assignments: 1 = glycolic acid, 2 = lactic acid, 3 = α-hydroxyisobutyric acid, 4 = α-hydroxybutyric acid, 5 = α-hydroxy-α-methylbutyric acid, 6 = malic acid, 7 = α-hydroxyglutaric acid, 8 = α-hydroxyisovaleric acid, 9 = α-hydroxyisocaproic acid, 10 = α-hydroxycaproic acid. Note: AU = arbitrary units.

5.4.2 *Detection of AHAs in ED Experiments*

During the course of an ED experiment, both AHAs and α-AAs can be synthesized via the Strecker/cyanohydrin pathways, where aldehydes (e.g. formaldehyde) or ketones serve as precursors (Figure 5.5),[39] depending on reaction conditions. The yields of these two product types are highly dependent on the pH of the aqueous phase and the abundance of NH_3 in the reaction medium.[40-41] Neutral-to-basic pH and high NH_3 concentrations favor AA synthesis, while acidic conditions and low NH_3 concentrations favor the formation of AHAs.[39] The pH values of the ED solutions in this study were slightly basic (generally between pH 8 and 9), and three of the four experiments contained significant amounts of NH_3 as a starting material, thus favoring AA synthesis. AHAs may still be observed as products formed via cyanohydrin synthesis in basic solutions, but typically in lower yields than α-AAs.[40]

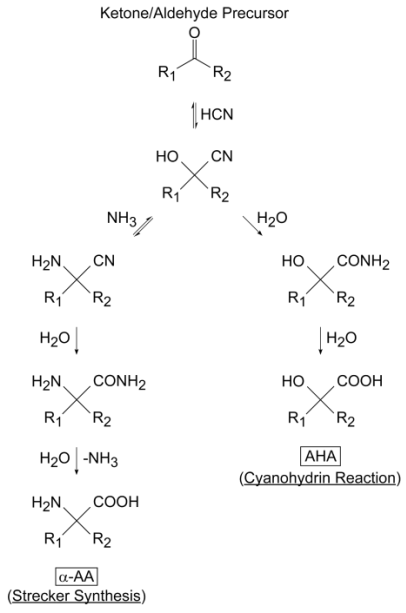


Figure 5.5. Schematic pathways showing the formation of α-AAs via the Strecker synthesis, and AHAs via the cyanohydrin reaction, starting from an aldehyde or ketone precursor.

The five most abundant protein α-AAs synthesized in the classic MU experiment were glycine > alanine > aspartic acid > valine > glutamic acid.[36] The corresponding AHAs are glycolic acid, lactic acid, malic acid, α-hydroxyisovaleric acid, and α-hydroxyglutaric acid, respectively. Of the range of AHAs that could be present in the complex mixtures generated by MU-type ED experiments, it is reasonable that these five would be among the most abundant, given the propensity for the formation of their AA analogs in Miller's classic experiment,[1, 36] as well as his H_2S,[42] and cyanamide[3] spark discharge experiments.

The two simplest AAs, glycine and alanine, are found in the greatest abundances in MU-type ED experiments.[26, 43] As a result, it is reasonable to expect glycolic acid and lactic acid to also be synthesized in MU-type ED reactions. Figure 5.4 gives an example of glycolic acid detection in one of the ED experiments performed here, while Figure 5.5 presents MRM chromatograms illustrating detection of lactic acid. The concentrations of glycolic acid and lactic acid were the highest of the AHAs investigated in this study, while those of the hydroxydicarboxylic acid species were typically lower (Table 5.3).

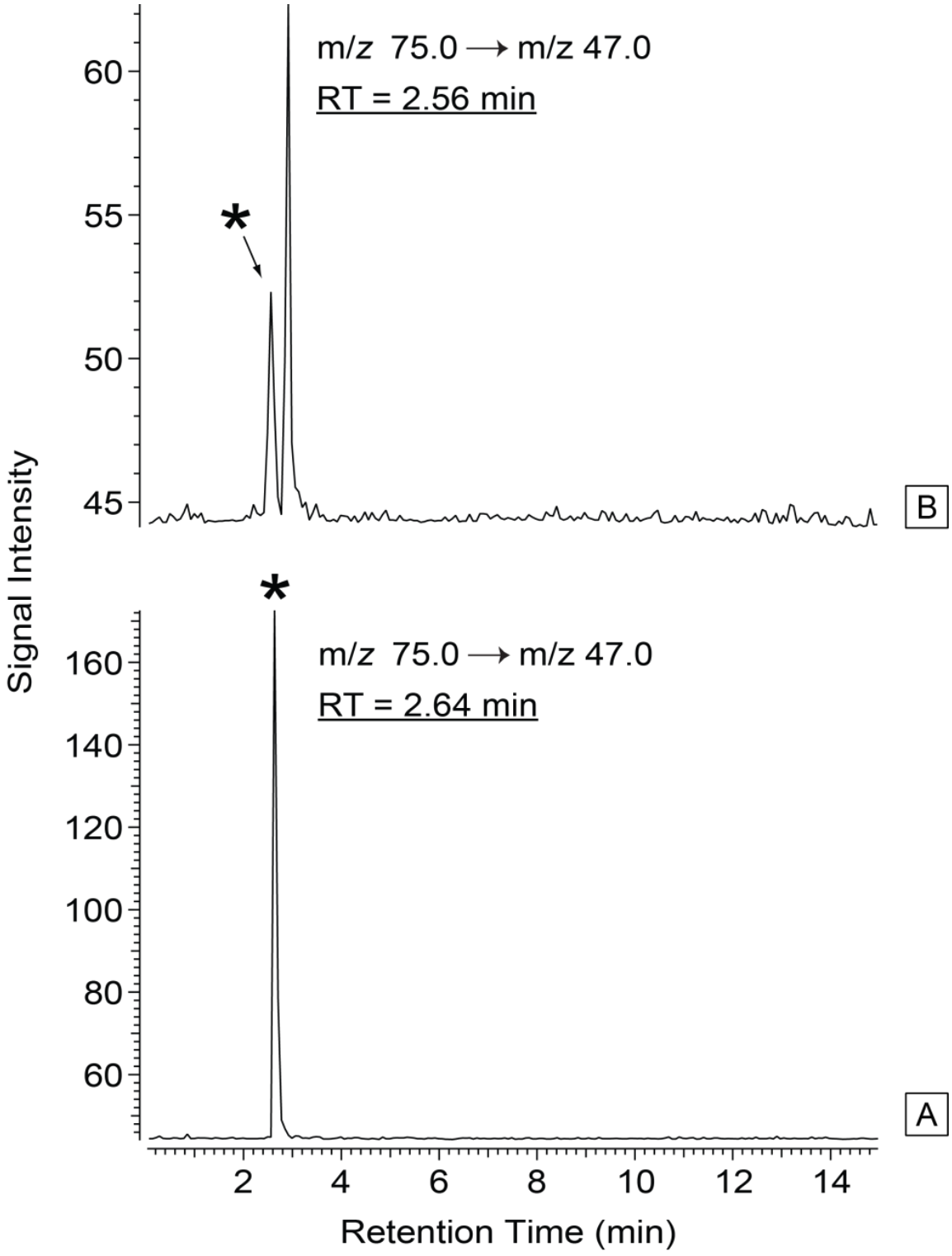


Figure 5.6. Individual 0 to 15 minute MRM mass chromatograms showing an example of glycolic acid detection as in A) a mixed AHA standard and B) ED Experiment #3. Asterisks denote peaks representing glycolic acid.

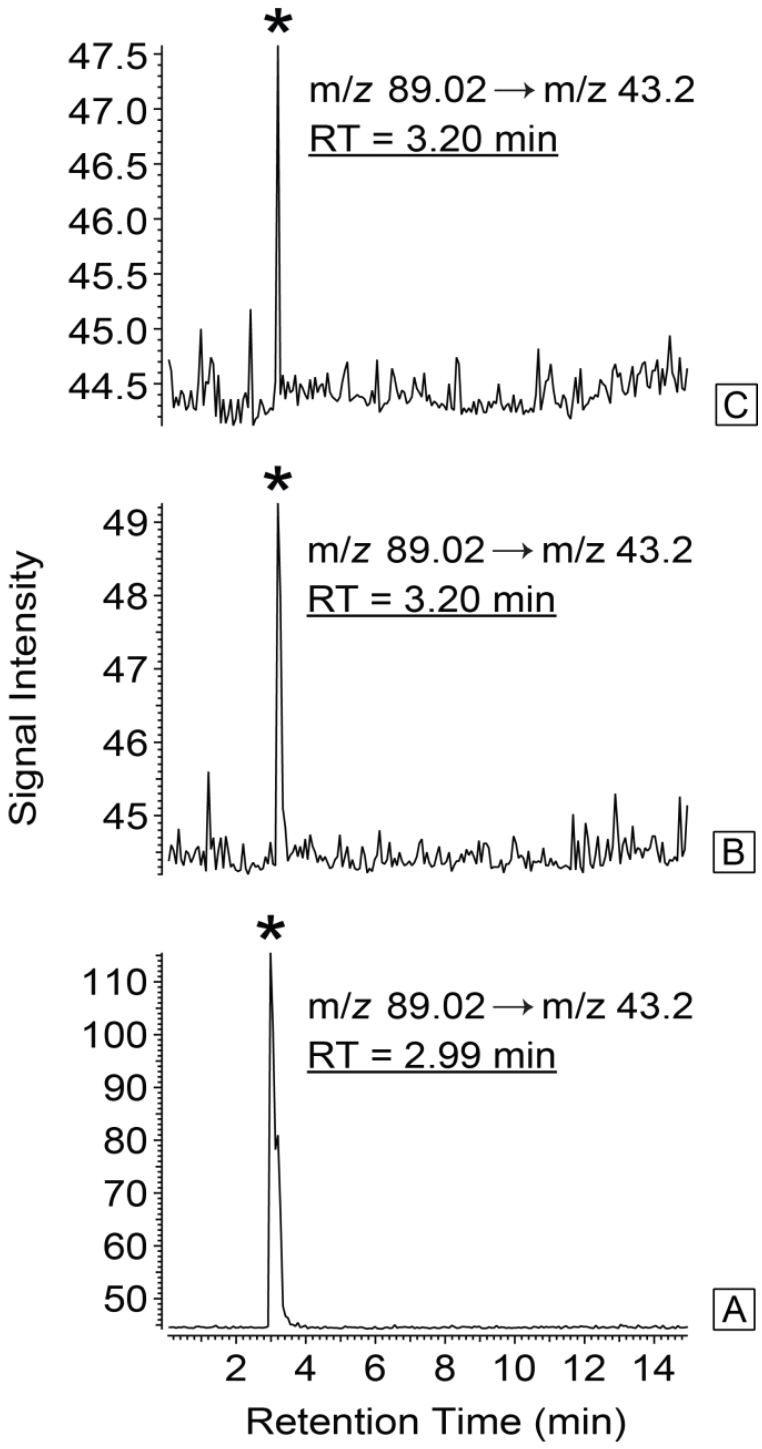


Figure 5.7. Example 0 to 15 minute MRM mass chromatograms demonstrating lactic acid detection in A) a mixed AHA standard, B) ED experiment #3, and C) ED experiment #4. Asterisks denote lactic acid. Small differences in analyte retention times were observed for samples and standards, due to the complexity of the samples. Injection of sample spiked with analyte standard confirmed peak identification in cases where analyte retention times in samples deviated from that of standards.

Based on the m/z 133.01 → 71.1 transition, malic acid concentrations were generally below the LOQ of 0.45 µM (Figure 5.6 and Table 5.3). However, malic acid was produced at a concentration greater than the LOQ in ED Experiment #3, and was detected above the LOQ in ED Experiment #1. Samples from ED Experiment #1, however, were concentrated 10x prior to analysis. Therefore, the quantity of malic acid in this concentrated sample exceeded the LOQ, yet the true concentration of malic acid in the discharge solution was 10 times lower, or 0.13 µM.

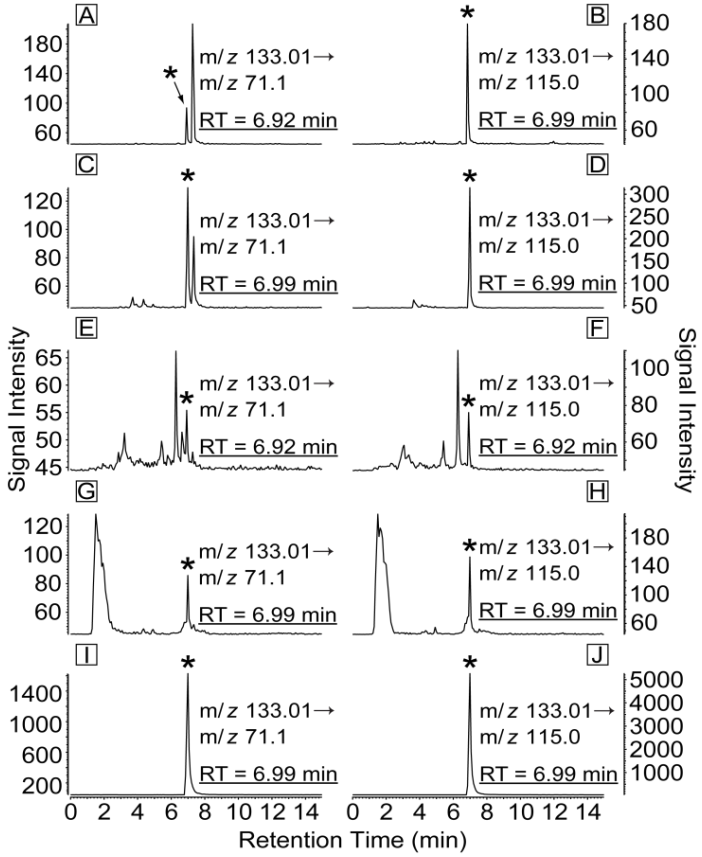


Figure 5.8. Individual 0 to 15 minute MRM mass chromatograms for both malic acid transitions developed in this study, demonstrating detection of this analyte in ED experiments. Here, I) and J) are from a mixed AHA standard, G) and H) are from ED Experiment #1, E) and F) are from ED Experiment #2, C) and D) are from ED Experiment #3, and A) and B) are from ED Experiment #4. Asterisks denote signals corresponding to malic acid.

The other dicarboxylic acid species analyzed for here, α-hydroxyglutaric acid, was detected in each of the 4 ED experiments (Figure 5.7). Yet, in none of these experiments was the concentration of this species above the LOQ. Therefore, the absolute concentration of α-hydroxyglutaric acid synthesized in these ED experiments is not reported in Table 5.3.

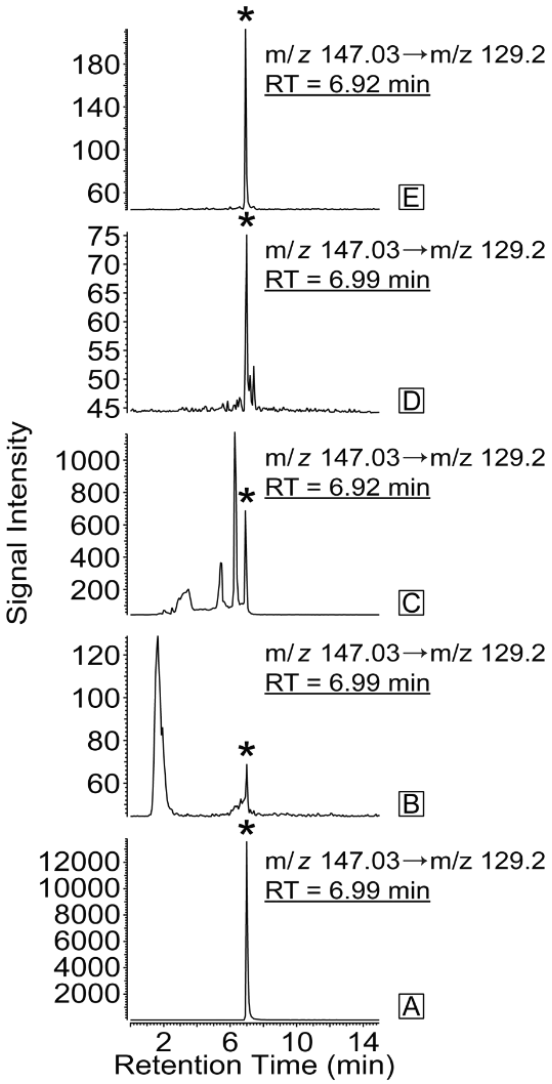


Figure 5.9. Individual 0 to 15 minute MRM mass chromatograms of α-hydroxyglutaric acid matching the m/z 147.03 → m/z 129.2 transition in a mixed AHA standard (A), ED experiment #1 (B), ED experiment #2 (C), ED experiment #3 (D), and ED experiment #4 (E). Asterisks denote peaks representing α-hydroxyglutaric acid.

Overall, abundances of AHAs synthesized in this study followed the general order of glycolic acid > lactic acid > malic acid > α-hydroxyglutaric acid (Table 5.3), which closely agrees with AA abundances measured here (Figure 5.8 and Table 5.4) and the classic MU experiment.[36] Glycine and alanine were generally the most abundant AAs formed, followed by lesser amounts of aspartic and glutamic acids. Valine was expected to be a significant α-AA, yet it was found at very low abundances in these samples. Low abundances of valine likely also explain why α-hydroxyisovaleric was not detected.

Table 5.3. AHA detection and quantitation in ED experiments from this work.

	Glycolic (μM); LOQ = 1.09 μM	Lactic (μM); LOQ = 2.38 μM	Malic (μM); LOQ = 0.45 μM	α-Hydroxyglutaric (μM); LOQ = 1.95 μM
ED #1[a]	<0.20	<0.43	1.3 ± 0.3	+
ED #2	31.5 ± 0.2	4.1 ± 0.3	+	+
ED #3	7.7 ± 0.1	3.8 ± 0.4	2.0 ± 0.4	+
ED #4	<0.20	+	+	+

[a]Analyte abundances for ED Experiment #1 represent those obtained from 10x concentrated samples.
[+]Analyte was detected, but concentrations did not exceed the LOQ.

Table 5.3 also shows that elevated quantities of glycolic acid were observed in ED Experiment #2. This finding can potentially be explained by the use of 0.225 M $NaHCO_3$ buffer in the reaction, leading to the introduction of supplemental CO_2 into the reaction flask, which may have been converted to additional glycolic acid precursors by the ED in the gas phase.

Prior to AA analysis, it is common practice to hydrolyze samples. Hydrolysis converts AA precursors, such as amino acid amides and amino nitriles to AAs, and releases bound AAs and AA precursors, increasing observed AA yields. However, this step was not necessary prior to AHA analysis. Because the pH values of ED solutions

were slightly basic, ester bonds readily hydrolyze, yielding individual AHAs that may have otherwise been involved in oligoesters[8] or depsipeptides.[9] These differences in sample preparation protocols can produce differences in analyte yields. Therefore, only general abundance comparisons between AAs and AHAs were made, but a comparison of AAs against AHAs could not be rigorously established.

5.4.3 *Complementary Detection of AAs in ED Experiments*

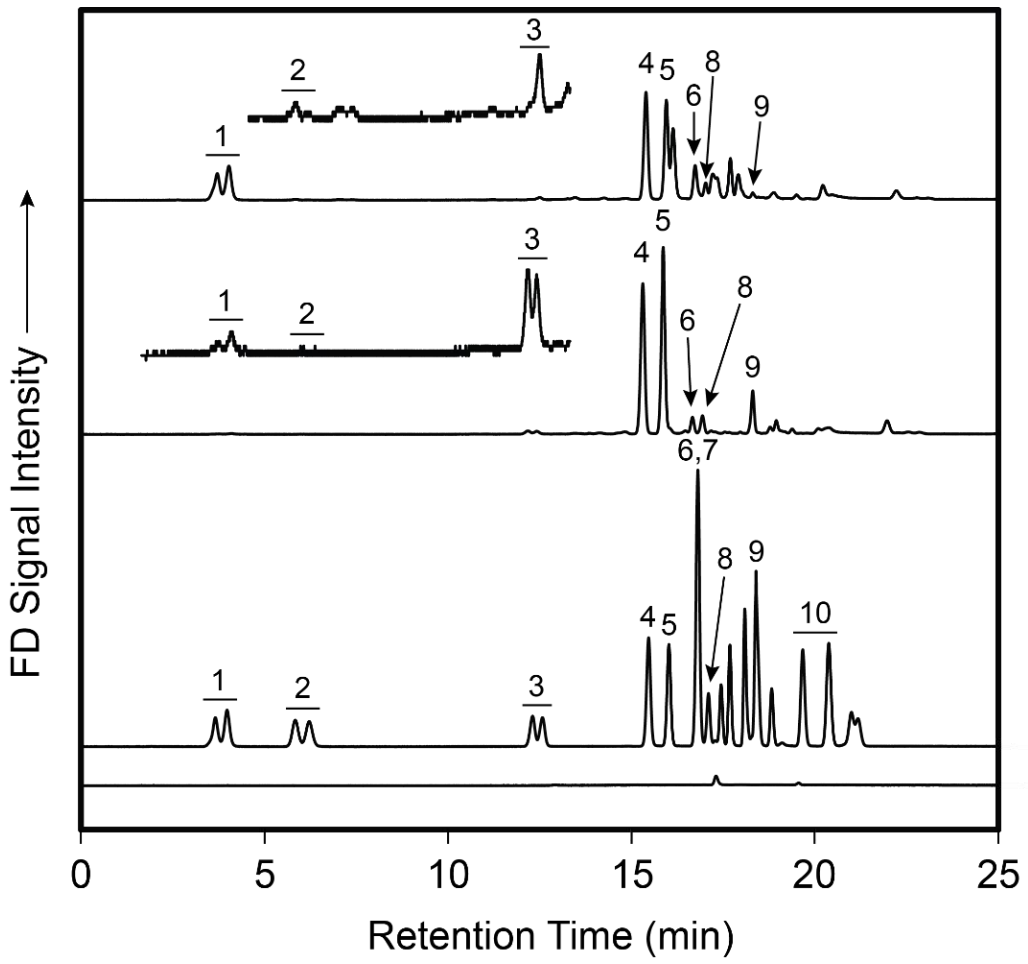


Figure 5.10. Example 0 to 25 minute fluorescence chromatograms of ED experiments #3 and #4, with standards of amino acids and amines, and a blank. Amino acid peak identifications: 1 = D,L-Asp; 2 = D,L-Glu; 3 = D,L-Ser; 4 = Gly; 5 = β-Ala; 6 = D-Ala; 7 = γ-ABA; 8 = L-Ala; 9 = α-ABA; 10 = D,L-Val. From bottom to top: water blank, amino acid and amine standard, ED Experiment #3, ED Experiment #4.The top two chromatograms include zoomed-in traces showing the signals for D,L-Asp, D,L-Glu, and D,L-Ser.

Table 5.4. Quantities of AAs. AA data collected from ED Experiments #3 and #4 are shown to exemplify the general trend of AA abundances observed: gly > ala > asp > glu.

AA	Molar Abundance (relative to Gly = 1)
Aspartic Acid	1.72×10^{-1}
Glutamic Acid	7.52×10^{-3}
Glycine	1
Alanine	2.67×10^{-1}

5.4.4 *Comparison to the Murchison Meteorite*

The Murchison meteorite contains both glycolic and lactic acids as the dominant AHAs, suggesting a robust synthesis for these species on the meteorite parent body.[16] Additional analyses have since been performed using GC-MS, with 19 hydroxy acids being detected and more than half of these being AHAs.[14] Of these 19 hydroxy acids, 5 were α-hydroxydicarboxylic acids, such as malic acid and α-hydroxyglutaric acid.[14] Beta- and γ-hydroxy acids have also been found in the Murchison meteorite, but at concentrations too low for proper quantitation.[44]

The findings of glycolic and lactic acids as the most abundant AHAs formed in these experiments, as well as smaller quantities of the α-hydroxydicarboxylic acids, malic acid and α-hydroxyglutaric acid, compare well to those obtained from previous studies of the Murchison meteorite. The protein α-AAs corresponding to the AHAs focused on here were also similarly detected in the ED solutions, and in the Murchison meteorite. Furthermore, these α-AA/AHA pairs were observed in similar orders of approximated abundances in the ED experiments executed in this work, which is consistent with their syntheses via the Strecker/cyanohydrin pathways suggested previously.[16]

5.5 Conclusions

This chapter reports the development of the first fast and direct method for the unambiguous detection and quantification of a large variety of AHAs in a single analytical run using UPLC/QqQ-MS. A total of 10 AHAs were targeted for separation using hexylamine ion-pairing chromatography. AHAs were subsequently detected via mass spectral transitions developed for each species. Detection limits for each AHA were determined to be in the nanomolar concentration range. The instrumental sensitivity was greatest for malic and α-hydroxyglutaric acids, the only two dicarboxylic acids among the tested analytes.

A series of MU-type ED experiments were performed using a variety of gas mixtures and aqueous solutions to study AHA syntheses under a range of possible primitive conditions. Among the experiments included in the cohort was the repeated Miller cyanamide spark discharge experiment. This work marks the first concerted efforts to analyze MU-type ED experiments for AHAs since Stanley Miller's explorations in the 1950s, where he used crude analytical techniques, relative to those techniques currently available. Through the use of powerful, modern analytical instrumentation, and the method developed here, this work confirms that the complex mixtures stemming from these experiments produced the AHAs glycolic acid, lactic acid, malic acid, and α-hydroxyglutaric acid.

Synthesis of AHAs was most significant in experiments containing N_2/CH_4 atmospheres in which the N_2 partial pressure exceeded that of CH_4, and the pH of the aqueous phase was slightly basic. Glycolic acid and lactic acid were produced in the greatest abundances, with lesser amounts of malic acid and α-hydroxyglutaric acid.

These findings indicate that future depsipeptide synthesis explorations, for the purpose of evaluating a possible route to prebiotic polypeptide formation, would be most realistically executed primarily using the building blocks glycine, alanine, glycolic acid, and lactic acid, along with smaller quantities of aspartic acid, glutamic acid, malic acid, and α-hydroxyglutaric acid.

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CHAPTER 6: ANALYSIS OF DIDEPSIPEPTIDES IN PREBIOTIC MIXTURES VIA TIME-NESTED ANALYTICAL TECHNIQUES

6.1 Abstract

The processes influencing the synthesis of the monomer organic species α-AAs and AHAs have proven to be robust, as evidenced by the detection of these species in prebiotic simulation experiments and carbonaceous meteorite studies. Although vast reports exist demonstrating the facile synthesis of AAs under primitive conditions, the knowledge of how AAs could have polymerized to form peptides via abiotic pathways remains sparse. It was recently reported that when subjected to wet-dry environmental cycling, a mixture of α-AAs and AHAs can readily generate depsipeptides, which contain both amide and ester linkages, becoming enriched in amide linkages over time. However, these results were obtained from dry-down experiments of a neat standard containing α-AA and AHA monomers co-dissolved in deionized water, where the monomer species were present at relatively high concentrations. Ideally, a low temperature prebiotic simulation experiment would be performed and the resultant solution analyzed for the presence of depsipeptides, to provide a more robust evaluation of the prebiotic plausibility of this chemistry. In this chapter, a multi-stage analytical platform has been developed using liquid chromatography, ion mobility spectrometry, and high resolution tandem mass spectrometry to enable the detection of a suite of didepsipeptides. Additionally, a series of ED experiments were performed and subjected to simulated environmental cycling to constrain the viability that the α-AAs and AHAs formed within these experiments could have co-polymerized, yielding their immediate polymerization products, didepsipeptides. The results of this investigation provide

evidence for the tentative identification of the glycolic acid-aspartic acid/malic acid-glycine dimers, and the malic acid-aspartic acid dimer. The overarching implication of the findings detailed in this chapter is that co-polymerization of AAs and AHAs within a complex mixture generated from a prebiotic simulation experiment, generates some of the same species that have been reported in the literature using neat standard solutions, but in lower yields.

6.2 Introduction

It has been well documented that protein building blocks are readily generated under a variety of imitated primordial Earth conditions.[1-8] However, far fewer published reports exist demonstrating the synthesis of the hydroxy acid analogs of such building blocks using mimicked early Earth conditions.[2, 9] And it is the co-polymerization of these monomers under prebiotically plausible conditions that remains an uncertainty in the prebiotic chemistry community.

The work discussed in Chapter 4 of this thesis and in the literature[8] has discovered a pathway by which simple peptides can be generated using simulated primitive conditions and the plausible prebiotic condensing reagent cyanamide. However, in this study, the formation of DKP was a favored product. More recently, Forsythe and Yu et al.[10] provided evidence that a mixture of AAs and AHAs can yield depsipeptides when subjected to dry/wet cycling to mimic day/night conditions. Such a process was proposed as a possible alternative prebiotic peptide synthetic pathway that may otherwise help mitigate significant DKP formation.

The approach to the experiments reported by Forsythe and Yu et al.[10] warrants further evaluation. First, neat mixtures of AAs and AHAs were prepared by dissolution of

these monomers in deionized water. Concentrations of monomers ranged from 1 mM, to 100 mM. The solutions were then subjected to drying and wetting periods before analysis. This is likely an oversimplification of a plausible prebiotic scenario as it is unlikely the primitive oceans, or tidal pools, were composed neat mixtures of AAs and AHAs at relatively high concentrations. It is more likely, however, that water reservoirs on the early Earth would have contained a cornucopia of vastly diverse molecules and ionic species, which can greatly complicate the chemical reactions that take place within an aqueous medium. Additionally, as was demonstrated by the work in Chapter 5 of this thesis, the types of monomers needed to produce depsipeptides are typically synthesized at concentrations on the order of a few-, to tens of micromolar in prebiotic simulation experiments, as opposed to tens of millimolar concentrations.

Ideally, a more accurate representation of a possible prebiotic environment would be investigated to rigorously evaluate the likelihood that such depsipeptide synthesis may be possible under primitive conditions. The solutions produced by these experiments could then undergo environmental cycling in an attempt to form depsipeptides. This sample mixture should then be analyzed for didepsipeptides, the immediate co-polymerization products of AAs and AHAs, to provide a baseline assessment of the possibility of generating simple depsipeptides in mimicked early Earth environments.

Developing an analytical method for depsipeptides would be of interest for more than just the work presented here and the greater prebiotic chemistry community. Depsipeptides are a keenly studied class of species possessing a number of intriguing biological ramifications. For example, depsipeptides are actively searched for and studied

for their potential use as antibacterial-,[11-16] anticancer-,[17-21] antivirus-,[22-26], antifungus-,[27-31] and anti-inflammatory-,[32-35] agents.

Select depsipeptide analytical methods have been previously developed. For example, Stokvis et al.[36] analyzed human plasma for the tridecadepsipeptide Kahalalide F using LC-MS/MS. The depsipeptide aplidine was detected in plasma and urine samples from rats, also using LC-MS/MS.[37] Li and Chan[38] developed and validated an LC-QqQ-MS method to detect the depsipeptide romidepsin in rat and human plasma. Kahalalide R and Kahalalide S, two cyclic depsipeptides, were detected in the marine mollusk *Elysia* grandifolia, a sea slug, using a quadrupole-TOF MS/MS approach.[39] Detection of such larger species (Kahalalide F: 1477.87 g mol^{-1}, aplidine: 1110.34 g mol^{-1}, romidepsin: 540.70 g mol^{-1}, Kahalalide R: 1519.91 g mol^{-1}, Kahalalide S: 1535.91 g mol^{-1}) is enabled by greater interaction of the target analyte with the stationary phase of the LC column, providing efficient chromatographic separation before MS analysis. This is contrary to smaller analytes, which tend to exhibit decreased interaction with reversed phase stationary phases, leading to co-elution and a greater propensity for ion suppression in the mass spectrometer ion source. This, in turn, can impact the ability of the mass spectrometer to correctly quantify the analyte.

There has been considerably less focus on the analysis of small depsipeptides, didepsipeptides in particular. Forsythe and Yu et al.[10] reported the use of IMS-MS/MS to detect and sequence depsipeptides, typically on the order of tetramers-to-hexamers (~300-400 Da), but with some examples of detection of depsipeptides as large as 14-mers, and as small as 2-mers. However, Forsythe and Yu et al.[10] did not implement a chromatographic component to the analytical platform implemented, which could help

mitigate ion suppression, as previously stated. In fact, chromatographic separation of didepsipeptides has not been reported since Wasielewski and Hoffmann[40] used paper chromatography to separate didepsipeptide diastereomers nearly half a century ago.

The work detailed in this chapter will address the combined needs of developing an analytical method that incorporates LC separation for the detection of didepsipeptides in complex mixtures, and evaluating the plausibility of depsipeptide synthesis in prebiotic mixtures. The analytical method developed here uses a combination of UPLC, TWIMS, and TOF-MS, as well as MS/MS. The goal of this analytical work is to provide separation of target didepsipeptide analytes in both the liquid and the gas phase, as well as mass analysis and identity confirmation via CID experiments. Analysis of depsipeptide product ions provides a second benefit of facilitating sequencing of tentatively detected didepsipeptides. A series of ED experimental samples underwent mimicked environmental cycling and the products were analyzed for didepsipeptides, the immediate co-polymerization products of AAs and AHAs.

6.3 Materials and Methods

6.3.1 *Chemicals and Reagents*

The water used for the work reported in this chapter was Nanopure Diamond (18.2 MΩ cm). All sample handling tools, including glassware, were washed with water and wrapped in aluminum foil before heating overnight at 500 °C, in air. Unless otherwise stated, all reagents used in this study were purchased from Acros Organics, Alfa Aesar, Chem-Impex International, Fluka Analytical, J.T. Baker, MP Biomedicals, and Sigma-Aldrich with purities ranging from 97 % to 99.99 %.

Stock solution of AAs and AHAs (10^{-3} M) were made by dissolving individual AA and AHA crystals in a solution of 5 mM hexylamine (Sigma-Aldrich, purity = 99%) in water, and then combining for analysis. Two eluents were used for UPLC-Q-TWIMS-TOF-MS analysis: (A) 5 mM hexylamine in water and (B) 90:10 methanol:10 mM NH_4OAc in water. Reagent (A) was prepared by dissolving hexylamine in water to make the solution 5 mM in hexylamine. Reagent (B) was prepared by dissolving NH_4OAc in water, to make the solution 10 mM in NH_4OAc, and adjusting the pH to 8.5 using 7 N NH_3 in methanol, prior to adding to LC-MS grade methanol.

The solution used for daily calibrations of the mass spectrometer was 0.5 mM sodium formate in 90:10 isopropanol:water. First, a 0.1 M solution of aqueous NaOH was made. Second, a 5 mM sodium formate solution was prepared by mixing 1 mL of 0.1 M aqueous NaOH, with 900 µL of deionized water, and 100 µL of formic acid prior to diluting to 20 mL with a 90:10 isopropanol:water solution. Finally, the 0.5 mM sodium formate solution in 90:10 isopropanol:water was generated by diluting 2 mL of the 5 mM sodium formate solution to 20 mL using 90:10 isopropanol:water.

Gases used for performing ED experiments were purchased from Airgas. ED experiments were performed using the following gases: ultra high purity Ar (99.99 % purity), ultra high purity CH_4 (99.99 % purity), ultra high purity N_2 (99.999 % purity), and anhydrous NH_3 (99.99 % purity).

Acid-vapor hydrolysis experiments were conducted by using ddHCl (99.999999 % (metals basis), 33 % min, Alfa Aesar, Product # 10990, Lot # M21C016). A stock solution of 11 M ddHCl was diluted to 6 M using Nanopure Diamond water, before performing acid-vapor hydrolysis of ED samples.

6.3.2 *Environmental Cycling Experiments with Model Mixtures*

Aqueous solutions containing the AAs glycine, alanine, aspartic acid, and glutamic acid, and the AHAs glycolic acid, lactic acid, malic acid, and α-hydroxyglutaric acid (each analyte was 1 mM) were prepared for environmental cycling experiments. To simulate a day cycle, solutions were allowed to dry down for 18 hours at 85 °C. A night cycle was then imitated by re-suspending the dried residues with 5 mM hexylamine in water. Sample supernatant was then acquired for analysis.

6.3.3 *ED Experiment #1*

ED experiment #1 was the repeated Miller cyanamide spark discharge experiment. The duplication of the cyanamide experiment has been detailed in the literature[8] and in Chapter 4 of this thesis. Consequently, the experimental protocol used to reproduce the cyanamide experiment will not be restated here. However, it should be noted that 10x concentrated solutions were also generated for analysis by drying down 20 mL aliquots of raw discharge solution, prior to bringing the dried residues back up in 2 mL of water.

6.3.4 *ED Experiment #2*

A mixture of 100 mmHg CH_4 and 200 mmHg N_2 was sparked in a 1 hour on/1 hour off cycle for 99 hours, over a 100 mL solution of 0.225 M aqueous $NaHCO_3$ that was adjusted to pH 8.7. At the completion of the experiment, samples were collected and frozen until analyzed. Full details regarding the execution of this ED experiment, and others detailed in this chapter, are based on the protocol outlined in the literature[41] and in Chapter 2 of this thesis.

6.3.5 *ED Experiment #3*

An ED experiment based on previous work[3] was conducted using a 0.225 M NH_3/NH_4Cl-buffered aqueous phase, and a N_2/CH_4 atmosphere. A 100 mL solution of 0.05 M ammonium chloride and 105 mmHg anhydrous NH_3 (to make the solution 17.5 mM in $\sum NH_3$ ($NH_3 + NH_4^+$), with a final pH of 8.7) were introduced into a 3 L reaction flask fitted with 2 tungsten electrodes. Nitrogen was added to the flask at a partial pressure of 300 mmHg, and CH_4 was added to the flask at a partial pressure of 110 mmHg. The ED was imparted across the electrodes over a 96-hour time period in a 1 hour on/off cycle.

6.3.6 *ED Experiment #4*

A final ED experiment was carried out, identical to ED Experiment #3, except for the use of a 200 mmHg N_2/200 mmHg CH_4 gas mixture, and the introduction of 3.708 g of synthetic schreibersite ($(Fe, Ni)_3P$)[42, 43] into the aqueous phase. The inclusion of $(Fe, Ni)_3P$ allowed for the investigation of the impact of the phosphorus-containing mineral to the aqueous phase chemistry.

6.3.7 *Environmental Cycling of ED Samples*

Aliquots (250 µL) of each of the aforementioned ED solutions were allocated for environmental cycling. The raw discharge samples were placed in sterilized, glass vials prior to cycling, which consisted of mimicking a daytime/nighttime environment. Daytime environments were simulated by heating at 65 °C, 75 °C, 85 °C, or 95 °C for 18 hours, while leaving the vials open to allow evaporation. Nighttime environments were mimicked by reconstituting the residues with 250 µL of water, then capping the sample vials to prevent evaporative loss, and subsequently heating the samples at 65 °C for 5.5

hours. After the desired number of cycles had been implemented, the samples were brought back up in 250 µL of water prior to analysis.

Samples were exposed to 0 - 4 imitated environmental cycles. Aliquots of each ED solution were analyzed directly, to represent a control sample that did not experience any environmental cycling. A single cycle was defined as heating an open sample vial for 18 hours. Two cycles was defined as heating an open sample vial at the chosen temperature for 18 hours, then reconstituting in 250 µL of water and heating a capped sample vial at 65 °C for 5.5 hours, then re-evaporating the sample at the chosen temperature for 18 hours. Three cycles were executed by performing one additional set of 18 hour/5.5 hour heating to that conducted for the two cycle scenario. And the 4 cycle scenario was executed by performing two additional sets of 18 hour/5.5 hour heating to that conducted for the two cycle scenario. Aliquots of each ED solution were exposed to one environmental cycle at 65 °C, 75 °C, 85 °C, and 95 °C. Additional aliquots of each sample were designated for 2, 3, and 4 cycles where the 18 hour heating period was maintained at 85 °C.

6.3.8 *Acid-Vapor Hydrolysis Experiments*

To investigate if signals corresponding to preliminary didepsispeptide detection did indeed represent species containing an amide linkage, acid-vapor hydrolysis experiments were performed on ED samples. Complete details of the acid-vapor hydrolysis experimental protocol can be found elsewhere,[44] but will be briefly overviewed here. First, 100 µL aliquots of ED solutions were brought to dryness, under vacuum, in an open test tube. Second, the open test tubes were flame-sealed inside larger test tubes that contained 1 mL of 6 M ddHCl. Third, samples were heated at 150 °C for 3

hours. Fourth, the outer test tubes were opened and the inner tubes, containing the samples, were removed carefully. Fifth, the extracted test tubes were dried under vacuum to evaporate any residual 6 M ddHCl that may be present after heating. Sixth, samples were reconstituted with 100 µL H_2O, before vortexing, and centrifugation to separate insoluble material. Lastly, the supernatant of the reconstituted sample was acquired for analysis.

6.3.9 *Mobility Survey via UPLC-Q-TWIMS-TOF-MS*

A Waters ACQUITY UPLC coupled to a Synapt G2 high definition mass spectrometer (Waters, Milford, MA, USA) operated in negative ion mode was used for didepsipeptide analysis in both the hydrolyzed and unhydrolyzed ED extracts.

The chromatographic method for the elution of didepsipeptides was optimized using a 2.1 x 100 mm, 1.7 µm particle size Waters ACQUITY UPLC BEH C18 column. Hexylamine was used as the ion-pairing agent added to the aqueous eluent to facilitate chromatographic retention of target analytes. The use of the hexylamine ion-pairing agent has been reported elsewhere[45] but the specific details regarding its use for the work in this chapter will be detailed here. The mobile phases used were (A) 5 mM hexylamine in deionized water and (B) 90:10 methanol:10 mM ammonium acetate in deionized water. A typical LC gradient used during elution of target analytes was: 0-2 min, 0 % B, 2-6 min, 0-20 % B, 6-7 min, 20 % B, 7-8 min, 20-0 % B, 8-10 min, 0 % B. Eluent was flowed at a rate of 0.25 mL min^{-1}. The column was heated at 50 °C, samples were maintained at 5 °C, and the injection volume used was 10 µL.

A dual ESI system was utilized for lock mass correction purposes. The primary ESI source was used and operated according to the following conditions: capillary

voltage, 1.9 kV; sample cone voltage, 25 V; extraction cone voltage, 4 V; source temperature, 100 °C; desolvation gas (N_2) temperature, 400 °C; desolvation gas flow rate, 700 L hr^{-1}. A 0.5 mM sodium formate solution was used for daily calibration of the mass spectrometer. However, it remains possible that subtle drifts in the m/*z* scale can occur after initial calibration. Consequently, a reference ESI source was utilized to supply an independent signal of a leucine enkephalin standard. The reference ESI source was operated with identical settings to those of the primary ESI source, except that the reference ESI source used a capillary voltage of 2 kV. The TWIMS settings used were the following: trap wave velocity, 300 m s^{-1}; trap wave height, 4 V; IMS wave velocity, 400 m s^{-1}; IMS wave height, 20 V; transfer wave velocity, 188 m s^{-1}; transfer wave height, 2 V. The TOF analyzer was operated in V-optics mode, providing a mass resolution of approximately 20,000 based on the full width at half maximum peak of the deprotonated leucine enkephalin ion at m/*z* 554.2615. The detector setting was 2175 V. In all experiments, the mass range acquired across was m/*z* 50-600. The relevant pressures, in millibars (mb), were: backing, 3.13 mb; source, 1.3 x 10^{-3} mb; trap, 2.22 x 10^{-2} mb; IMS, 3.77 mb; TOF, 1.01 x 10^{-6} mb.

After implementing the desired number of environmental cycles to the relevant samples, aliquots were subjected to mobility survey analyses for didepsipeptides via UPLC-Q-TWIMS-TOF-MS. Didepsipeptides were identified, first by accurate mass UPLC-MS runs to search for peaks associated with the accurate masses of didepsipeptide $[M-H]^-$ ions within a mass window of 10 mDa. AA and AHA monomers (Table 6.1) considered in potential didepsipeptides (Table 6.2) included glycine, alanine, aspartic acid, glutamic acid, glycolic acid, lactic acid, malic acid, and α-hydroxyglutaric acid.

Table 6.1. List of AA and AHA monomers considered in potential didepsipeptides, including monomer elemental formulas, neutral masses, and theoretical masses in negative ion mode.

Monomers	Elemental Formula	Neutral Mass	Theoretical $[M-H]^-$
Glycine (G)	$C_2H_5NO_2$	75.0320	74.0242
Glycolic Acid (g)	$C_2H_4O_3$	76.0160	75.0082
Alanine (A)	$C_3H_7NO_2$	89.0477	88.0399
Lactic Acid (a)	$C_3H_6O_3$	90.0317	89.0239
Aspartic Acid (D)	$C_4H_7NO_4$	133.0375	132.0297
Malic Acid (d)	$C_4H_6O_5$	134.0215	133.0137
Glutamic Acid (E)	$C_5H_9NO_4$	147.0532	146.0453
α-Hydroxyglutaric Acid (e)	$C_5H_8O_5$	148.0372	147.0293

Table 6.2. List of didepsipeptides targeted during analysis.

Potential Didepsipeptides	Elemental Formula	Neutral Mass	Theoretical $[M-H]^-$
gG/Gg	$C_4H_7NO_4$	133.0375	132.0297
gg	$C_4H_6O_5$	134.0215	133.0137
aG/Ga, gA/Ag	$C_5H_9NO_4$	147.0532	146.0453
ag/ga	$C_5H_8O_5$	148.0372	147.0293
aA/Aa	$C_6H_{11}NO_4$	161.0688	160.0610
aa	$C_6H_{10}O_5$	162.0528	161.0450
gD/Gd, dG/Dg	$C_6H_9NO_6$	191.0429	190.0352
dg/gd	$C_6H_8O_7$	192.0269	191.0192
dA/Da, aD/Ad, gE/Ge, eG/Eg	$C_7H_{11}NO_6$	205.0586	204.0508
da/ad, eg/ge	$C_7H_{10}O_7$	206.0426	205.0348
eA/Ea, aE/Ae	$C_8H_{13}NO_6$	219.0743	218.0665
ea/ae	$C_8H_{12}O_7$	220.0583	219.0505
dD/Dd	$C_8H_{11}NO_8$	249.0484	248.0406
dd	$C_8H_{10}O_9$	250.0324	249.0247
dE/De, eD/Ed	$C_9H_{13}NO_8$	263.0641	262.0563
ed/de	$C_9H_{12}O_9$	264.0481	263.0403
eE/Ee	$C_{10}H_{15}NO_8$	277.0798	276.0719
ee	$C_{10}H_{14}O_9$	278.0638	277.0560

To facilitate mobility survey of target didepsipeptides, an inclusion list was used in combination with a dynamic exclusion function. The inclusion list was utilized to preferentially select for target ions with accurate masses corresponding to didepsipeptides, prior to CID fragmentation during the course of MS/MS runs. Dynamic

exclusion was implemented in such a manner that upon detection of ions on the inclusion list, any masses within a 100 mDa mass window of the mass in question would be subsequently excluded for 5 seconds. Therefore, data for masses outside of that 100 mDa mass window can be acquired within this 5 second time span.

After this screening step, MS/MS experiments were conducted using quadrupole precursor ion selection followed by TWIMS separation of analytes in the gas phase, and CID of target precursor ions in the transfer cell. Ar gas was used for transfer cell CID experiments, and the CID energy implemented was 30 eV (lab frame).[B] Tandem MS experiments involved acquiring didepsipeptide product ion spectra over a 10 second retention time window, centered on the elution times of target precursor ions, while using a mass tolerance of 10 mDa for matching product ions.

6.4 Results and Discussion

6.4.1 *Environmental Cycling Experiments in Model Mixtures*

To evaluate the ability of mimicked environmental cycling to facilitate didepsipeptide formation of the chosen monomers, mobility survey analyses were performed on a monomer solution and its dry-down products. Chromatographic separation of AA and AHA monomer species is shown in Figure 6.1. The use of hexylamine ion-pairing chromatography enabled C_2 and C_3 AAs to be almost completely

[B]There are two types of reference frames adopted by the mass spectrometry community as it pertains to the energy used for CID during tandem MS experimentation: 1) lab reference frame and 2) center of mass reference frame. Lab reference frame refers to the energy imparted during CID, depending on the voltages used and electric fields applied within the mass spectrometer ion optics. Center of mass reference frame refers to the energy imparted during CID, depending on the mass of the target gas. Consequently, lab reference frame is instrument-specific, while center of mass reference frame is more applicable to numerous instruments. More detailed discussions about lab reference frame and center of mass reference frame can be found in Rodgers and Armentrout,[45] Sleno and Volmer,[47] and Forsythe et al.[48]

resolved. A similar trend was observed in the case of C_2 and C_3 AHAs. The dicarboxylic acid species were well-resolved from the C_2 and C_3 monomers. Similarly, dicarboxylic acid AAs were fully resolved from dicarboxylic acid AHAs. Much like the C_2 and C_3 AAs, the dicarboxylic acid AAs have different retention times, but overlap slightly. The dicarboxylic acid AHAs also co-elute to some degree, despite having unique retention times. Signal intensity for the dicarboxylic acid monomers was higher than that of the C_2 and C_3 monomers, likely due to greater ionization efficiency of those species containing two carboxylic acid groups.

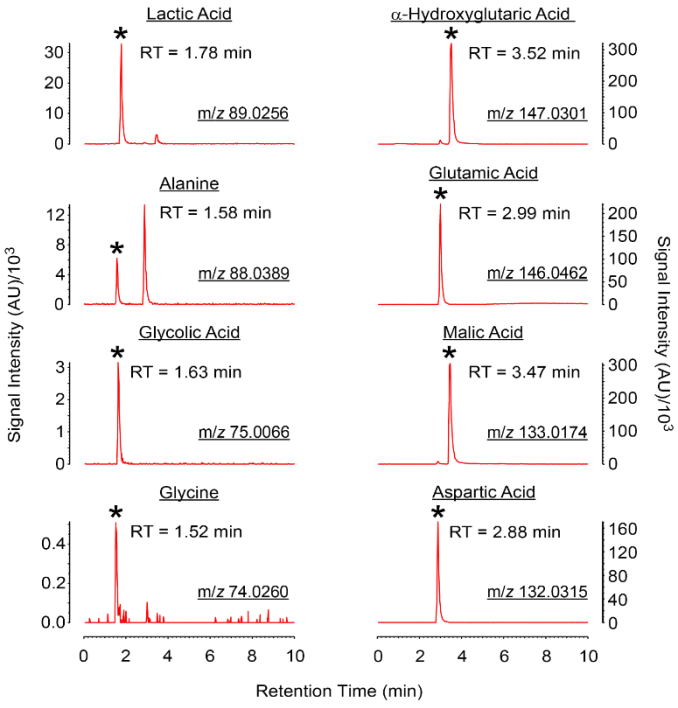

Figure 6.1. Extracted UPLC-MS chromatograms of a combined mixture of AAs and AHAs, where each analyte is present at a concentration of 50 µM. Asterisks represent target monomer analyte $[M-H]^-$ ions. In the trace for alanine, the peak at RT = 2.88 min, is a product of in-source fragmentation of aspartic acid, which yields a fragment ion with an m/z value very near to the $[M-H]^-$ ion of alanine (see Figure 6.3.A as an example), hence the co-elution of this fragment ion with the aspartic acid $[M-H]^-$ ion. Here, RT = retention time, and AU = arbitrary units.

Separation of AA and AHA monomers in the gas phase was also investigated by TWIMS-MS (Figure 6.2) during the same analysis as described above. Much like during UPLC of the monomers, the dicarboxylic acid species were well-separated from the C_2 and C_3 monomers. The C_2 monomers experienced some overlap in their respective drift times, as did the C_3 monomers. The analogs, aspartic acid and malic acid possessed very similar drift times, as expected, due to their comparable structural characteristics. Likewise, glutamic acid and α-hydroxyglutaric acid were also separated in the gas phase, but drift times were similar.

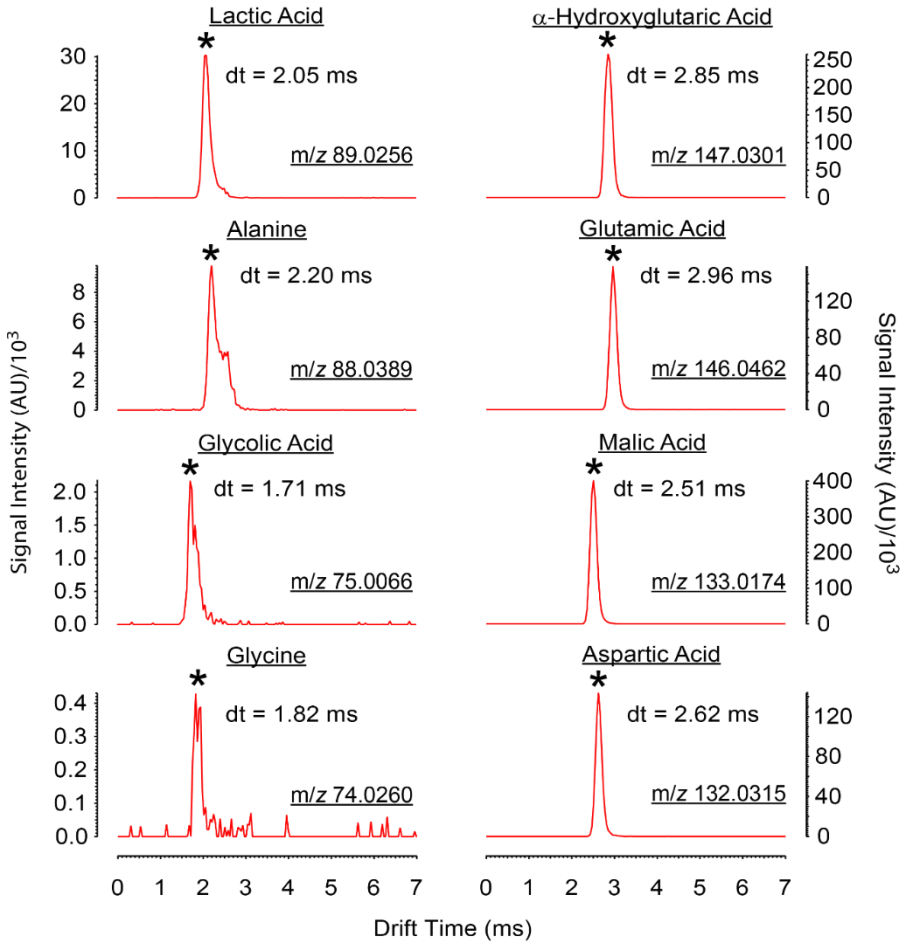

Figure 6.2. Extracted TWIMS chronograms of a combined mixture of AAs and AHAs, where each analyte is present at a concentration of 50 µM. Asterisks represent target monomer analyte $[M-H]^-$ ions. Here, dt = drift time and AU = arbitrary units.

To evaluate the fragmentation patterns of monomer species, MS/MS experiments were performed on the compounds listed in Table 6.1. During the execution of mobility survey MS/MS experiments, only the species with precursor ions with the highest signal intensities were selected for CID in the transfer cell, as these species were the most likely to produce sufficiently abundant product ions to be detected. Consequently, only the monomers with an LC signal intensity $\geq\sim10^5$ (i.e. dicarboxylic acid AAs and AHAs) were selected for fragmentation (Figure 6.3). In the case of these monomers, the use of 30 eV in the transfer cell was sufficient to produce abundant product ions without depleting the precursor ions. It is worth noting that upon fragmenting aspartic acid, a product ion of m/*z* 88.0430 is readily produced (Figure 6.3.A), which has a Δm of 3.1 mDa from the $[M-H]^-$ ion of alanine. This product ion is the cause of the chromatographic peak observed at 2.88 minutes, when screening for the $[M-H]^-$ ion of alanine.

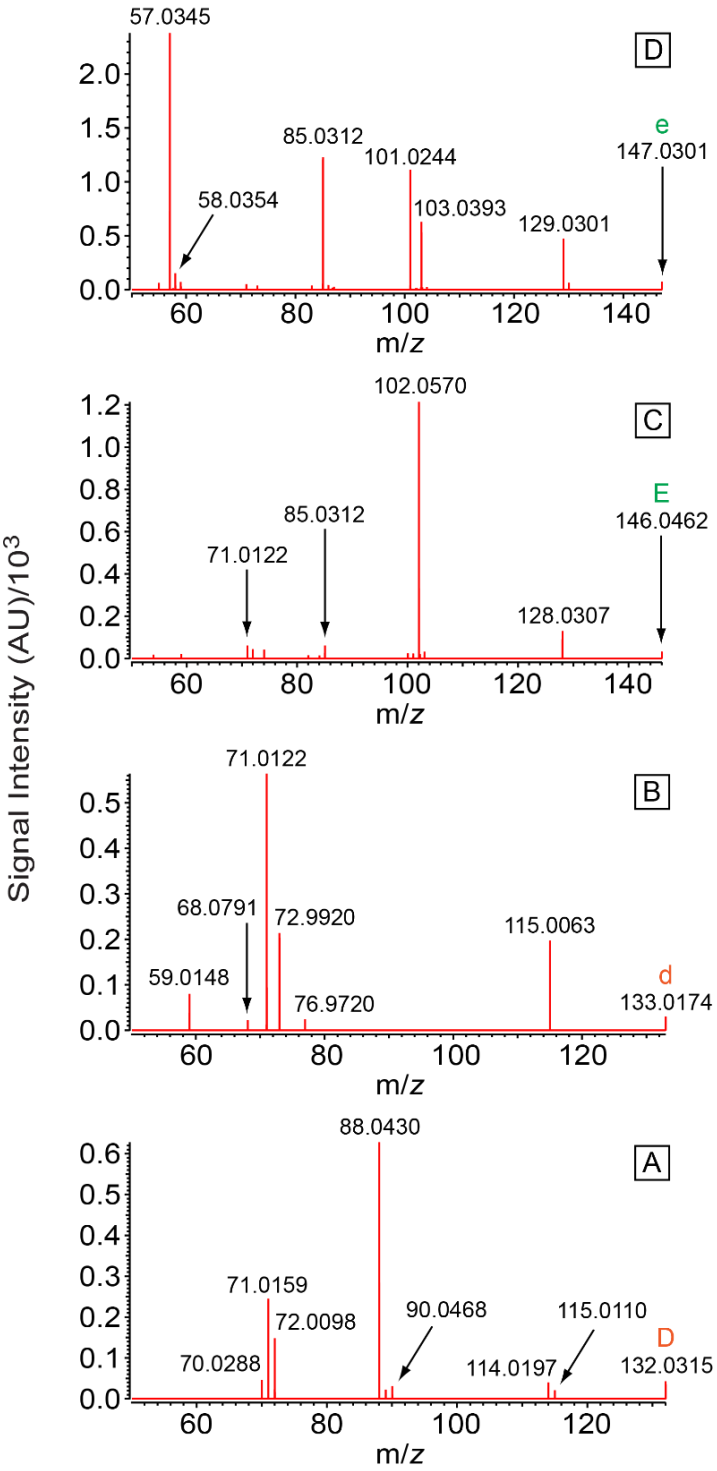

Figure 6.3. MS/MS spectra of the following monomers: A) aspartic acid, B) malic acid, C) glutamic acid, and D) α-hydroxyglutaric acid. In this figure, the $[M-H]^-$ ions are denoted by their respective single letter codes, as described in Table 6.1. Additionally, analog species are noted in matching colors. For example, the orange, capitalized letter "D" represents the aspartic acid $[M-H]^-$ ion and the orange, lower cased letter "d" represents the analog malic acid $[M-H]^-$ ion.

After drying down a solution of AA and AHA monomers, where each analyte was present at a concentration of 10^{-3} M, an aliquot was acquired for dimer analysis, following a procedure identical to the one used for the monomers. All of the possible didepsipeptides that could have been formed from environmental cycling (Table 6.2) were searched for in the analysis. Complete baseline separation of target didepsipeptides via UPLC (Figure 6.4) and TWIMS (Figure 6.5) is inherently challenging. To explain, there are 52 possible species (48 dimers and 4 monomers) that can be detected at a total of 18 m/*z* values associated with the $[M-H]^-$ precursor ions of interest. When searching for these 52 total possible species, 29 peaks were detected during UPLC-MS analysis (Figure 6.4), however, UPLC-MS analysis, alone, is not sufficient to confirm if these 29 peaks necessarily represent target didepsipeptides. MS/MS analysis of these 29 peaks would enable further investigation of the species contributing to these detected signals. Of these, 25 experienced some degree of chromatographic co-elution. Of the 25 co-eluting peaks, 8 shared the exact same retention time. It is worth noting that in Figure 6.4.E, there are 6 detectable peaks, when only one analyte is targeted at m/z 160.0610, demonstrating the complex nature of the sample, containing several isobaric species. It should also be recognized in Figure 6.4.F, that dilactic acid appears to have been generated at trace levels after one mimicked environmental cycle. One potential explanation for this is the pH of the solution. The monomers were initially dissolved in the starting LC eluent (100% 5 mM hexylamine), which has a pH ~11. When reconstituted after drying down, the solution may have maintained sufficient basicity to cleave the simple ester bond of dilactic acid, yielding the lactic acid monomers. Alternatively, dilactic acid could have quickly polymerized into larger species.

These UPLC-MS results show that the LC approach taken provided retention and moderate separation of target analytes. However, to further improve analyte separation, post-ionization TWIMS was attempted. During ion mobility experimentation, 19 peaks were detected across the 18 m/z values of interest (Figure 6.5). Only 2 peaks were not completely resolved via TWIMS-MS, as can be seen by the near-baseline resolution of the 2 peaks in Figure 6.5.J, both of which were detected at the m/z 205.0353 ion.

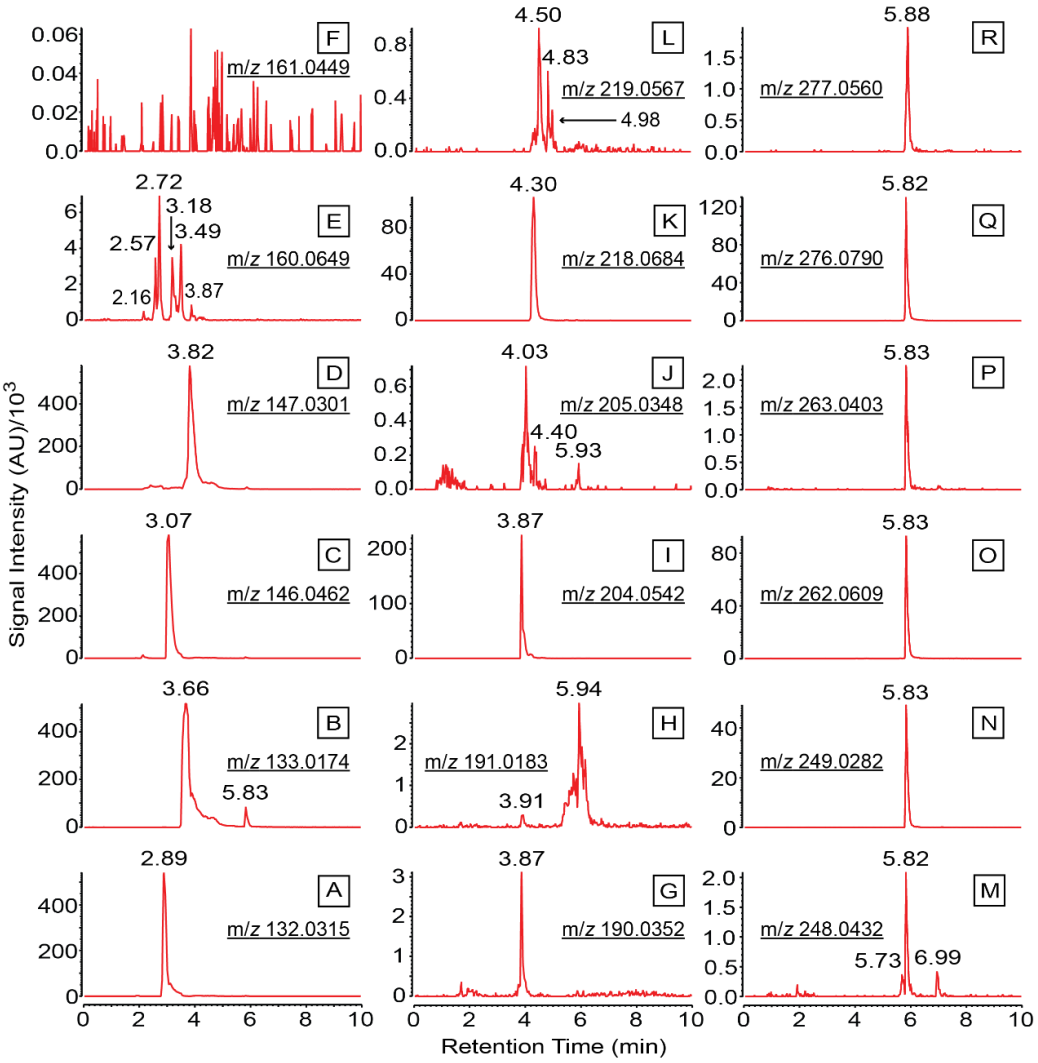

Figure 6.4. Extracted UPLC-MS chromatograms of a 10^{-3} M combined AA/AHA solution that underwent one simulated environmental cycle. Here, AU = arbitrary units.

Individual panels are labeled for association with references made to the figure, in the text.

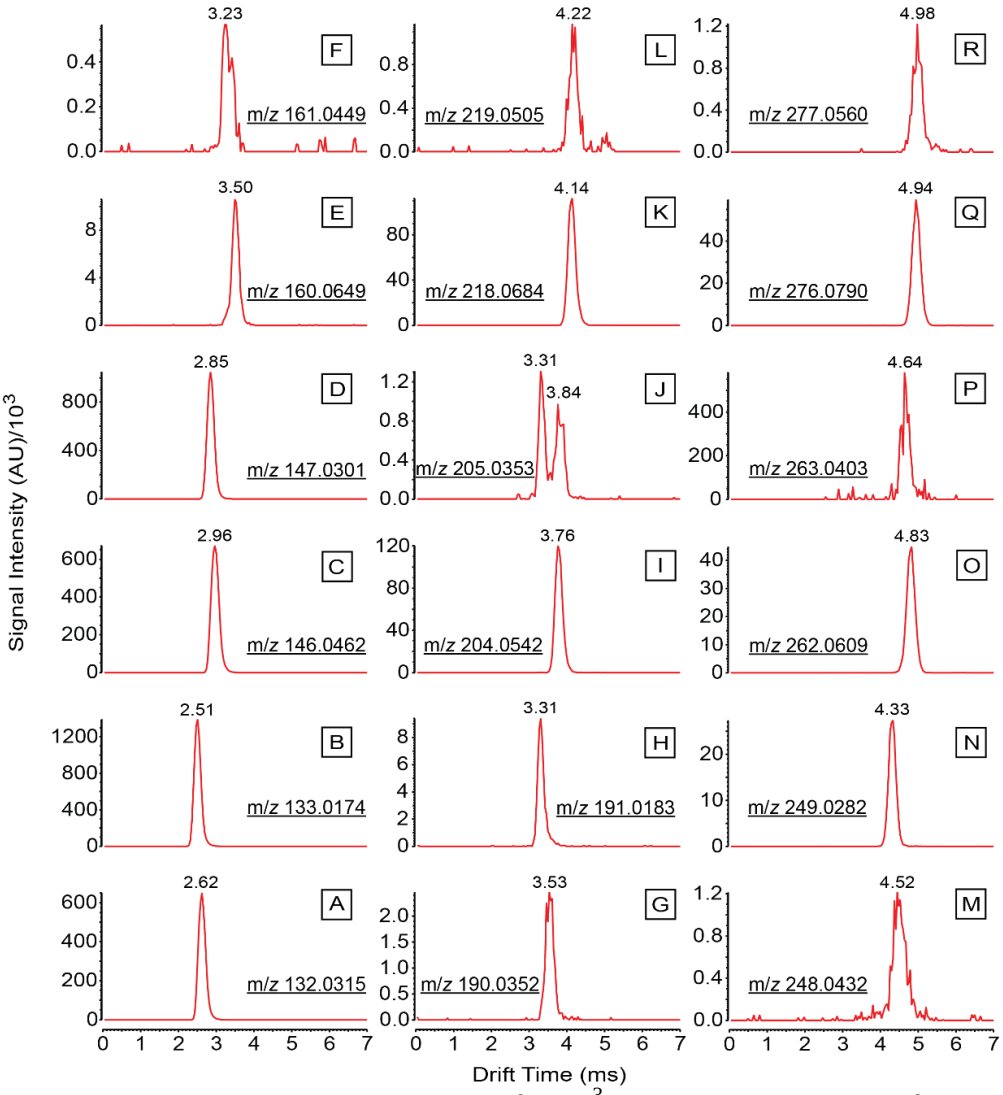

Figure 6.5. Extracted TWIMS chronograms of a 10^{-3} M combined solution of AAs and AHAs that underwent one simulated environmental cycle. Here, AU = arbitrary units. Panels are labeled for association with references made to the figure, in the text.

Only half of the target m/z values were associated with species present in the mixture at sufficiently high abundances (precursor ion intensity of ≥~10^4) for MS/MS analysis (Figure 6.6): m/z 132.0315, m/z 133.0124, m/z 146.0462, m/z 147.0301, m/z 204.0542, m/z 218.0684, m/z 249.0282, m/z 262.0609, and m/z 276.0718. These were identified based on the fragmentation patterns of each $[M-H]^-$ ion, as shown below.

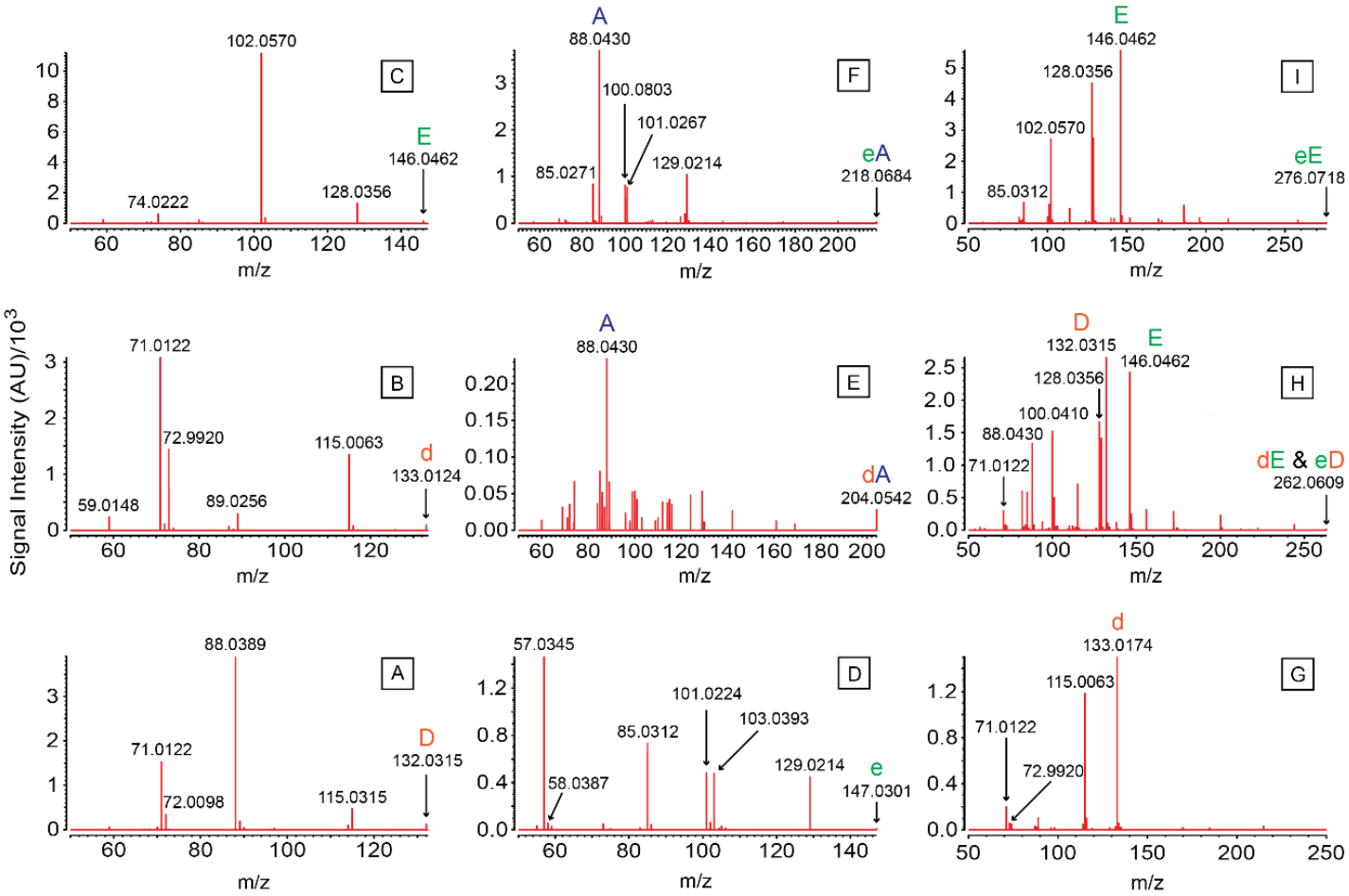

Figure 6.6. MS/MS spectra of A) m/z 132.0315, B) m/z 133.0124, C) m/z 146.0462, D) m/z 147.0301, E) m/z 204.0542, F) m/z 218.0684, G) m/z 249.0282, H) m/z 262.0609, and I) m/z 276.0718, from the 10^{-3} M mixture of AAs and AHAs that underwent one environmental cycle. Legend: orange, capitalized letter "D" represents aspartic acid; orange, lower-cased letter "d" represents malic acid; green, capitalized letter "E" represents glutamic acid; green, lower-cased letter "e" represents α-hydroxyglutaric acid; blue, capitalized letter "A" represents alanine. Here, AU = arbitrary units.

Figure 6.6.A shows the fragmentation pattern for m/z 132.0315, which corresponds to either aspartic acid, or the glycolic acid-glycine dimer. The product ion spectrum of this precursor ion was nearly identical to that of aspartic acid (Figure 6.3.A), demonstrating that the signal for m/z 132.0315 arising from the mixture undergoing one cycle, was aspartic acid. If it were the glycolic acid-glycine dimer, one would expect to see the $[M-H]^-$ ion of either glycine (m/z 74.0242), or glycolic acid (m/z 75.0082). However, neither of these species were observed in the product ion spectrum.

Figure 6.6.B shows the fragmentation pattern for m/z 133.0124, which corresponds to either malic acid, or the glycolic acid dimer. The product ion spectrum of this precursor ion was nearly identical to that of malic acid (Figure 6.3.B), demonstrating that the signal for m/z 133.0124 arising from the mixture undergoing one cycle, was malic acid. If it were the glycolic acid dimer, one would expect to see the $[M-H]^-$ ion of glycolic acid (m/z 75.0082). However, this species was not observed.

Figure 6.6.C shows the fragmentation pattern for m/z 146.0462, which corresponds to glutamic acid, the lactic acid-glycine dimer, or the glycolic acid-alanine dimer. The product ion spectrum of this precursor ion was nearly identical to that of glutamic acid (Figure 6.3.C), demonstrating that the signal for m/z 146.0462 arising from the mixture undergoing one cycle, was glutamic acid. If it were the lactic acid-glycine dimer, one would expect to see the $[M-H]^-$ ion of either glycine (m/z 74.0242) or lactic acid (m/z 89.0239). The $[M-H]^-$ ion of lactic acid is not observed, but there appears to be a very small signal for the $[M-H]^-$ ion of glycine. However, this ion is also observed in the product ion spectrum for glutamic acid, therefore the possibility that the lactic acid-glycine dimer is contributing to the signal at m/z 146.0462 is ignored. If it were the

glycolic acid-alanine dimer, one would expect to see the $[M-H]^-$ ion of either glycolic acid (m/z 75.0082) or alanine (m/z 88.0399). However, these species were not observed in the product ion spectrum.

Figure 6.6.D shows the fragmentation pattern for m/z 147.0301, which corresponds to either α-hydroxyglutaric acid, or the lactic acid-glycolic acid dimer. The product ion spectrum of this precursor ion was nearly identical to that of α-hydroxyglutaric acid (Figure 6.3.D), demonstrating the signal for m/z 146.0462 arising from the mixture undergoing one cycle was α-hydroxyglutaric acid. If it were the lactic acid-glycolic acid dimer, one would expect to see the $[M-H]^-$ ion of either glycolic acid (m/z 75.0082), or lactic acid (m/z 89.0239). However, these species were not observed.

Figure 6.6.E shows the fragmentation pattern for m/z 204.0542, which corresponds to either the α-hydroxyglutaric acid-glycine dimer, the malic acid-alanine dimer, the lactic acid-aspartic acid dimer, or the glycolic acid-glutamic acid dimer. A relatively pronounced signal for the m/z 88.0430 product ion was observed, which represents the $[M-H]^-$ ion of alanine. This evidence demonstrated that the signal for m/z 204.0542 arising from the mixture undergoing one cycle, was the malic acid-alanine dimer. If it were the α-hydroxyglutaric acid-glycine dimer, one would expect to see the $[M-H]^-$ ion of either α-hydroxyglutaric acid (m/z 147.0293), or glycine (m/z 74.0242). However, these species were not observed in the product ion spectrum. If it were the lactic acid-aspartic acid dimer, one would expect to see the $[M-H]^-$ ion of either lactic acid (m/z 89.0239), or aspartic acid (m/z 132.0297) acid. However, these species were not observed in the product ion spectrum. If it were the glycolic acid-glutamic acid dimer,

one would expect to see the $[M-H]^-$ ion of either glycolic acid (m/z 75.0082), or glutamic acid (m/z 146.0453) acid. However, these species were not observed.

Figure 6.6.F shows the fragmentation pattern for m/z 218.0684, which corresponds to either the α-hydroxyglutaric acid-alanine dimer, or the lactic acid-glutamic acid dimer. A relatively pronounced signal for the m/z 88.0430 product ion was observed, representing the $[M-H]^-$ ion of alanine. This demonstrated that the signal for m/z 218.0684 arising from the mixture undergoing one cycle, was the α-hydroxyglutaric acid-alanine dimer. If it were the lactic acid-glutamic acid dimer, one would expect to see the $[M-H]^-$ ion of either lactic acid (m/z 89.0239), or glutamic acid (m/z 146.0453) acid. However, these species were not observed in the product ion spectrum.

Figure 6.6.G shows the fragmentation pattern for m/z 249.0282, which corresponds to dimalic acid. A relatively pronounced signal for the m/z 133.0174 product ion was observed, which represents the $[M-H]^-$ ion of malic acid. This evidence demonstrated that the signal for m/z 249.0282 arising from the mixture undergoing one cycle, was dimalic acid. Further evidence for the formation of dimalic acid can be seen when looking at the additional product ions in the spectrum: m/z 115.0063, m/z 72.9920, and m/z 71.0122. These product ions closely match those for malic acid (Figure 6.6.B), and were likely the result of further fragmentation of malic acid monomer.

Figure 6.6.H shows the fragmentation pattern for m/z 262.0609 (although the precursor ion has been depleted in this product ion spectra), which corresponds to either the malic acid-glutamic acid dimer, or the α-hydroxyglutaric acid-aspartic acid dimer. Relatively pronounced signals for the m/z 132.0315 and m/z 146.0462 product ions were observed, which represent the $[M-H]^-$ ions of aspartic acid and glutamic acid,

respectively. This evidence demonstrated that the signal for m/*z* 262.0609 arising from the mixture undergoing one cycle, was from both the malic acid-glutamic acid dimer and the α-hydroxyglutaric acid-aspartic acid dimer. Further evidence for the formation of the α-hydroxyglutaric acid-aspartic acid dimer can be seen when looking at the additional product ions in the spectrum, namely the m/*z* 88.0430 and m/*z* 71.0122 ions. These product ions closely match those for aspartic acid (Figure 6.6.A), and were likely the result of further fragmentation of aspartic acid monomer.

Figure 6.6.I shows the fragmentation pattern for m/*z* 276.0718, which corresponds to the α-hydroxyglutaric acid-glutamic acid dimer. A relatively pronounced signal for the m/*z* 146.0462 product ion was observed, which represents the $[M-H]^-$ ion of glutamic acid. This evidence demonstrated that the signal for m/*z* 276.0718 arising from the mixture undergoing one cycle, was from the α-hydroxyglutaric acid-glutamic acid dimer. Further evidence for the formation of the α-hydroxyglutaric acid-glutamic acid dimer can be seen when looking at the additional product ions in the spectrum: m/*z* 128.0356, m/*z* 102.0570, and m/*z* 85.0312. These product ions closely match those for aspartic acid (Figure 6.6.C), and were likely the result of further fragmentation of aspartic acid.

Based on MS/MS experiments, the most likely candidate species matching the ions with sufficiently high signal intensities for CID are aspartic acid, malic acid, glutamic acid, α-hydroxyglutaric acid, malic acid-alanine dimer, α-hydroxyglutaric acid-alanine dimer, dimalic acid, malic acid-glutamic acid dimer, α-hydroxyglutaric acid-aspartic acid dimer, and α-hydroxyglutaric acid-glutamic acid dimer. A summary of the analysis of the environmental cycling experiment is presented in Table 6.3**,** Table 6.4, and Table 6.5.

Table 6.3. List of monomers detected during environmental cycling experiments with model mixtures.

Monomers	Theoretical $[M-H]^-$	Experimental $[M-H]^-$	$[M-H]^-$ (mDa)
G	74.0242	74.0260	1.8
g	75.0082	75.0104	2.2
A	88.0399	88.0389	1.0
a	89.0239	89.0256	1.7
D	132.0297	132.0315	1.8
d	133.0137	133.0174	3.7
E	146.0453	146.0462	0.9
e	147.0293	147.0301	0.8

Table 6.4. Review of recorded UPLC, TWIMS, and MS metrics during analysis for dimers formed from environmental cycling experiments with model mixtures. All potential dimers are listed here, except for when MS/MS was possible. In which case, only the most probably sequences are listed (as explained in Table 6.5). Here, RT = retention time and dt = drift time.

Potential Didepsipeptides	Theoretical $[M-H]^-$	Experimental $[M-H]^-$	RT	dt	MS/MS Confirmation?
gG/Gg	132.0297	132.0315	2.89	2.62	No
gg	133.0137	133.0174	3.66, 5.83	2.51	No
aG/Ga, gA/Ag	146.0453	146.0462	3.07	2.96	No
ag/ga	147.0293	147.0301	3.82	2.85	No
aA/Aa	160.0610	160.0649	2.16, 2.57, 2.72, 3.18, 3.49, 3.87,	3.50	No
aa	161.0450	161.0449	N/A	3.23	N/A
gD/Gd, dG/Dg	190.0352	190.0352	3.87	3.53	No
dg/gd	191.0192	191.0183	3.91, 5.94	3.31	No
dA/Ad	204.0508	204.0542	3.87	3.76	Yes
da/ad, eg/ge	205.0348	205.0348	4.03, 4.40, 5.93	3.31, 3.84	No
eA/Ae	218.0665	218.0684	4.30	4.14	Yes
ea/ae	219.0505	219.0567	4.50, 4.83, 4.98	4.22	No
dD/Dd	248.0406	248.0432	5.73, 5.82, 6.99	4.52	No
dd	249.0247	249.0282	5.83	4.33	Yes
dE/Ed	262.0563	262.0609	5.83	4.83	Yes
ed/de	263.0403	263.0403	5.83	4.64	No
eE/Ee	276.0719	276.0790	5.82	4.94	Yes
ee	277.0560	277.0560	5.88	4.96	No

Table 6.5. List of dimers tentatively detected during environmental cycling experiments with model mixtures. Only those dimers for which product ion spectra were collected, are product ions given.

Dimers	Theoretical $[M-H]^-/y_1$	Experimental $[M-H]^-/y_1$	$[M-H]^-/y_1$ Δm (mDa)
aA/Aa	160.0610/-	160.0649/-	3.9/-
gD/Gd, dG/Dg	190.0352/-	190.0352/-	0/-
dg/gd	191.0192/-	191.0183/-	0.9/-
dA	204.0508/88.0399	204.0542/88.0430	3.4/3.1
da/ad, eg/ge	205.0348/-	205.0348/-	0/-
eA	218.0665/88.0399	218.0684/88.0430	1.9/3.1
ea/ae	219.0505/-	219.0567/-	6.2/-
dD/Dd	248.0406/-	248.0432/-	2.6/-
dd	249.0247/133.0137	249.0282/133.0174	3.5/3.7
dE, eD	262.0563/(132.0297, 146.0453)	262.0609/(132.0315, 146.0462)	4.6/(1.8, 0.9)
ed/de	263.0403/-	263.0403/-	0/-
eE	276.0719/146.0453	276.0790/146.0462	7.1/0.9
ee	277.0560/-	277.0560/-	0/-

6.4.2 *Environmental Cycling of ED Mixtures*

A wide variety of environmental cycling experiments were carried out to evaluate the circumstances under which didepsipeptides may be generated from raw ED solutions. This chapter will provide examples of data collected from the analysis of each of the 4 ED experiments performed, to provide a representative overview of the successes and challenges encountered when searching for didepsipeptides within prebiotic mixtures generated throughout this thesis.

The solution produced by ED experiment #1 was subjected to 4 environmental cycles of 85 °C dry-down for 18 hours/65 °C rehydration for 5.5 hours. The resulting mixtures were analyzed for didepsipeptides and compared to the findings of an identical analysis of a raw discharge solution from ED experiment #1, which did not undergo any environmental cycles. Evidence for the formation of the glycolic acid-aspartic acid dimer/malic acid-glycine dimer espousing from environmental cycling of the complex mixture from ED experiment #1 is shown in Figure 6.7. UPLC-MS analysis of these sample solutions showed that dimers were not present at the m/z 190.0352 ion in the raw discharge solution, which was not exposed to environmental cycling. However, a UPLC-MS peak for the m/z 190.0352 ion existed in each of the 4 cycled samples from ED experiment #1, at a retention time near that of the peak for m/z 190.0352 that resulted from a 10^{-3} M AA/AHA mixture that underwent 1 environmental cycling at 85 °C.

When examining the TWIMS chronograms resulting from these samples, there was not a detectable peak at m/z 190.0352 in the raw discharge solution, but there was in the samples that underwent 1, 2, 3, and 4 cycles. Additionally, these chronogram peaks

were very similar in drift time, to the drift time of the m/z 190.0352 peak resulting from the 10^{-3} M mixture of AAs and AHAs that experienced one environmental cycle at 85 °C.

It can be seen that as the number of cycles increased, the signal intensity for the m/z 190.0352 peak, in both the UPLC-MS and IMS data sets, decreased, indicating that a maximum abundance was achieved after 1 cycle. However, it cannot be distinguished which of the two potential dimers that share the m/z 190.0352 (or both) may have contributed to the observed peaks, as the signal intensity, and hence the analyte abundance, was too low for proper identification and sequencing via tandem MS. Additionally, didepsipeptide standards are difficult to synthesize and are commercially expensive, preventing such standards from being readily available for use in compound identification. As a result, the detection of glycolic acid-aspartic acid dimer/malic acid-glycine dimer is based only on retention time, drift time, and m/z matching with the species produced in the model mixtures.

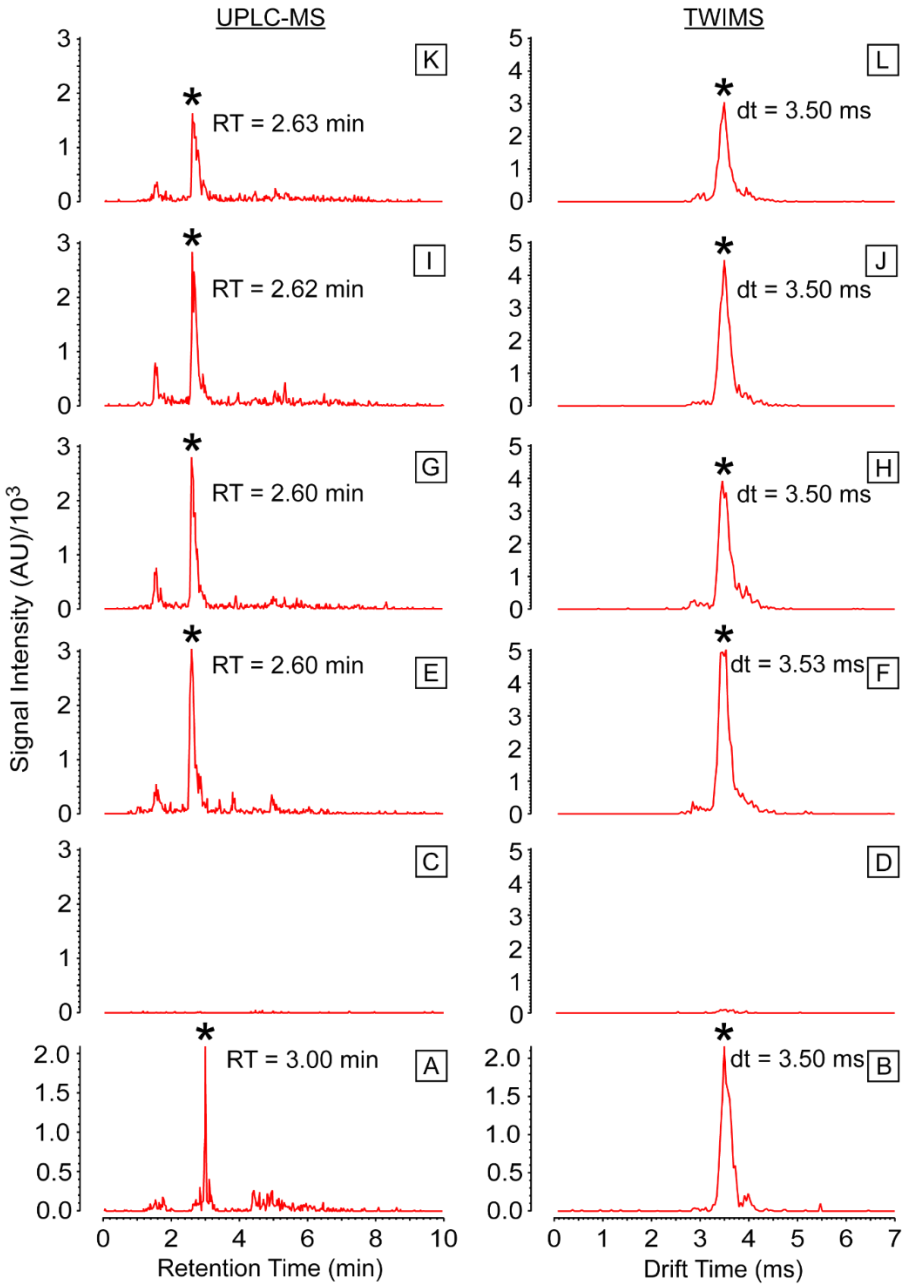

Figure 6.7. UPLC-MS (left) and TWIMS (right) chromatograms and chronograms, respectively, for m/z 190.0352. A) and B) are from the 10^{-3} M mixture of AAs and AHAs that underwent one environmental cycle. C) and D) are from the raw discharge solution of the ED experiment #1 (i.e. did not undergo cycling). E) and F) are from ED experiment #1 solution that underwent 1 cycle. G) and H) are from ED experiment #1 solution that underwent 2 cycles. I) and J) are from ED experiment #1 solution that underwent 3 cycles. K) and L) are from ED experiment #1 solution that underwent 4 cycles. Asterisks represent peaks used to tentatively identify the glycolic acid-aspartic acid dimer/glycine-malic acid dimer. Small differences in analyte retention times were observed for samples and dried down monomer standards due to the complexity of the samples.

The possibility that didepsipeptides were generated from the environmental cycling of sample solution from ED experiment #2 was explored, however, only weak evidence of dimer synthesis was obtained. For example, Figure 6.8 shows the UPLC-MS and TWIMS analysis of m/z 276.0790 (α-hydroxyglutaric acid-glutamic acid dimer) for ED experiment #2 after 0 cycles, 1 cycle at 65 °C, 1 cycle at 75 °C, 1 cycle at 85 °C, and 1 cycle at 95 °C. There was a small UPLC-MS peak at m/z 276.0790 for the ED experiment #2 solution that experienced 1 environmental cycle at 65 °C, which shared an identical retention time with the m/z 276.0790 peak associated with the mixture of dried down monomer standards. However, such a peak did not exist in the ED experiment #2 samples that underwent one cycle at 75 °C, 85 °C, or 95 °C.

The corresponding ion mobility chronograms of the discharge samples show peaks with drift times that were fairly dissimilar to that observed in a mixture of monomer standards that underwent one cycle at 85 °C. Furthermore, the ion mobility chronogram for the sample that underwent 1 cycle at 65 °C was nearly identical to those of the samples that underwent one cycle at 75 °C, 85 °C, and 95 °C, despite these latter samples not possessing a corresponding UPLC-MS peak.

Although there is one case in which UPLC-MS data suggests that perhaps the α-hydroxyglutaric acid-glutamic acid dimer may have been formed in small amounts, the inconsistency observed between the UPLC-MS data and the TWIMS data suggests it is unlikely this dimer was formed by the discussed cycling procedures. Lastly, the signal intensity of the m/z 276.0790 peak in Figure 6.8.E was insufficient to be selected for fragmentation. Therefore, the identity of this peak could not be explored further.

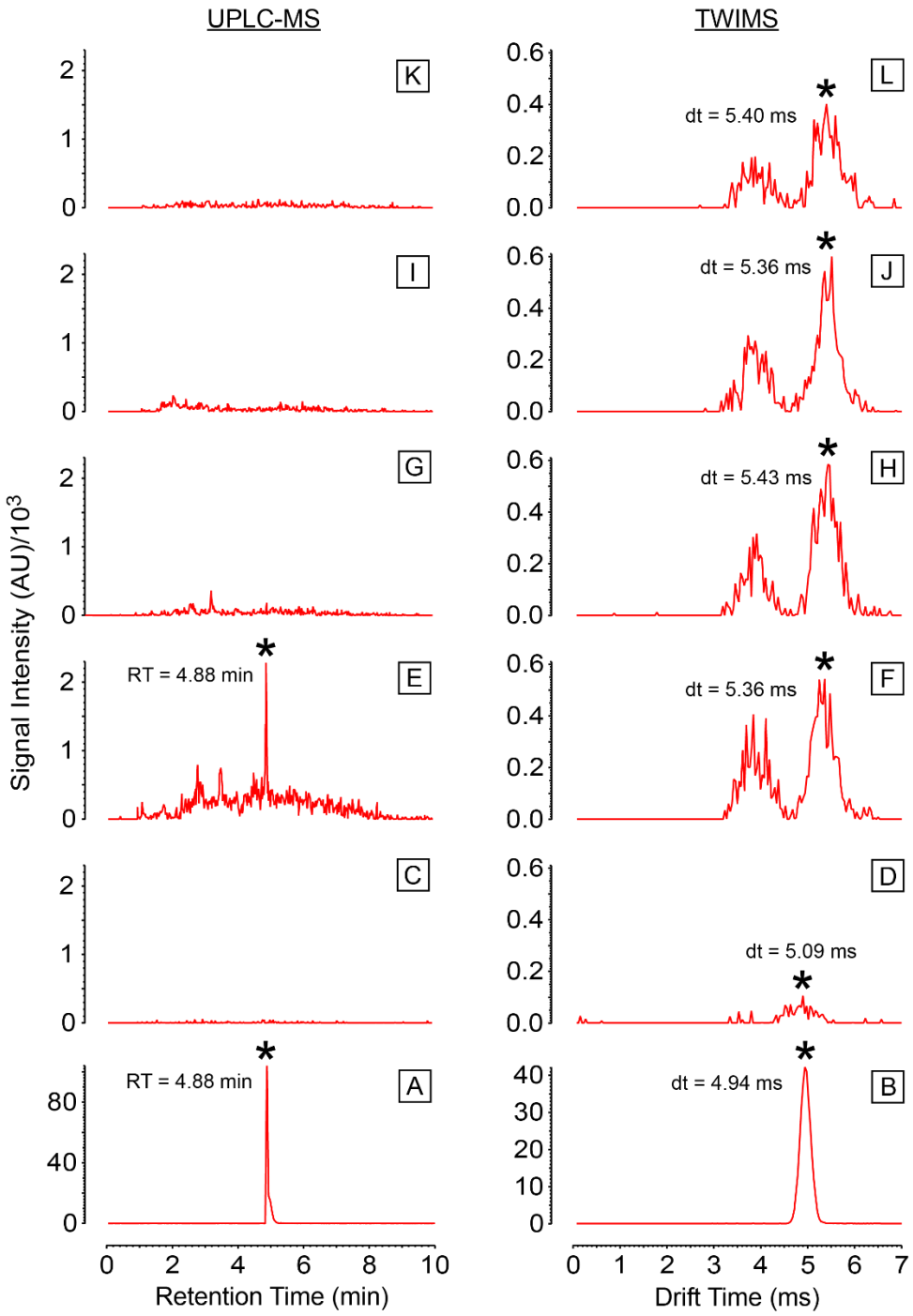

Figure 6.8. UPLC-MS (left) and TWIMS (right) chromatograms and chronograms, respectively, for m/z 276.0719. A) and B) are from the 10^{-3} M mixture of AAs and AHAs that underwent 1 environmental cycle. C) and D) are from the raw discharge solution of ED experiment #2 (i.e. did not undergo cycling). E) and F) are from ED experiment #2 solution that underwent 1 cycle at 65 °C. G) and H) are from ED experiment #2 solution that underwent 1 cycle at 75 °C. I) and J) are from ED experiment #2 solution that underwent 1 cycle at 85 °C. K) and L) are from ED experiment #2 solution that underwent 1 cycle at 95 °C.

The cycling of a discharge solution from ED experiment #3 at 65 °C, 75 °C, 85 °C, and 95 °C was also examined. When compared to a 10^{-3} M solution of AAs and AHAs that underwent one environmental cycle at 85 °C, there was evidence that the glycolic acid-aspartic acid dimer/malic acid-glycine dimer was present at m/z 190.0352 (Figure 6.9). The control sample of ED experiment #3 that underwent zero environmental cycles did not show a peak at m/z 190.0352 in either the UPLC-MS data (Figure 6.9.C), or the TWIMS data (Figure 6.9.D). However, such a signal existed in the samples from ED experiment #3 that underwent cycling, and was in close agreement with the expected retention time and drift time. The signal intensity for the $[M-H]^-$ ion at m/z 190.0352 is most intense in the case of cycling at 65 °C, perhaps suggesting that larger temperatures may have contributed to enhanced decomposition of the dimer at m/z 190.0352, possibly facilitating the formation of higher order depsipeptides. Due to the low $[M-H]^-$ ion signal intensity, MS/MS experiments could not be performed to determine the fragmentation pattern of this precursor ion. Consequently, the formation of the glycolic acid-aspartic acid dimer/malic acid-glycine dimer from environmental cycling of sample from ED experiment #3, can only be matched to the model solutions investigated earlier.

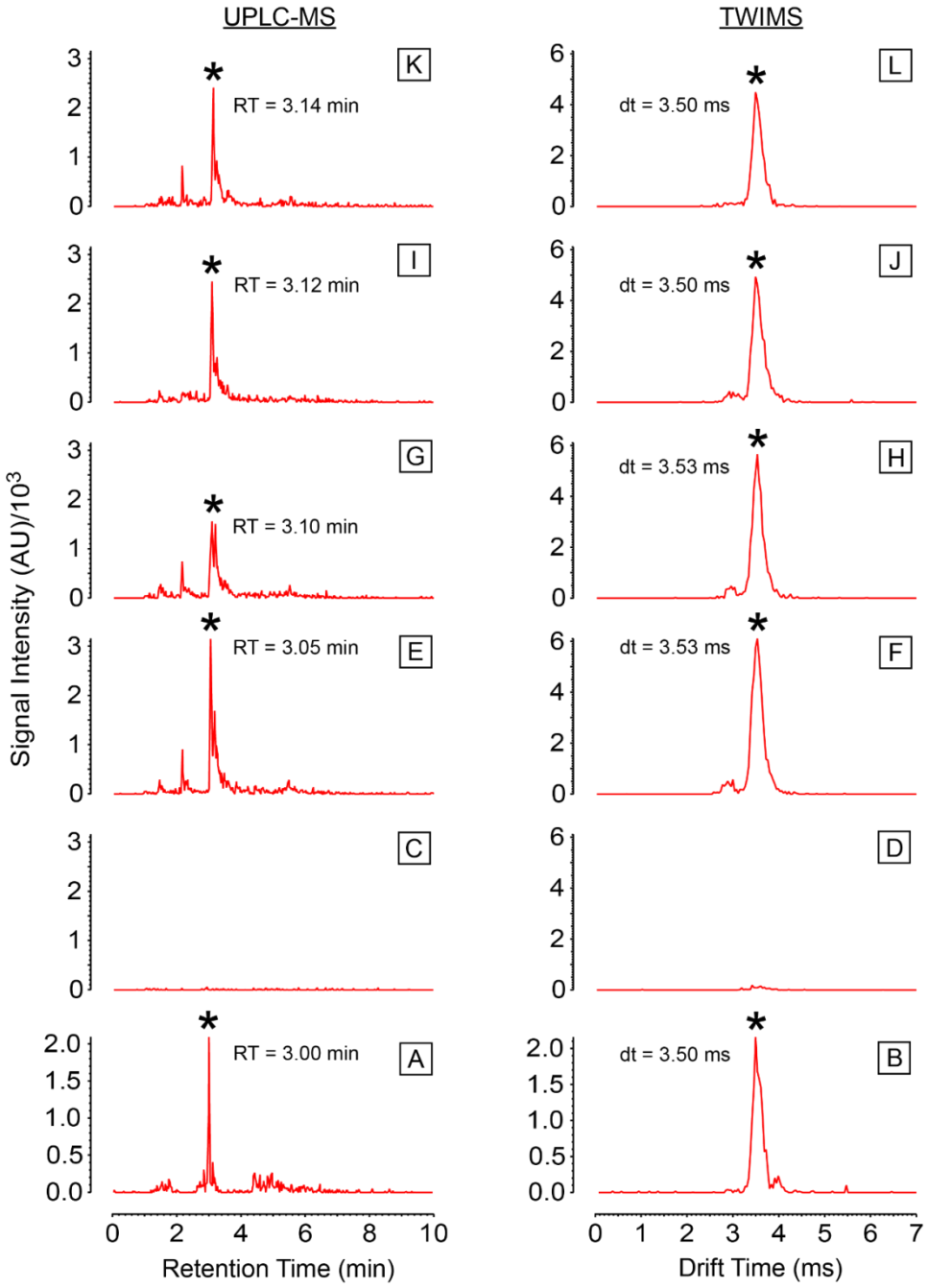

Figure 6.9. UPLC-MS (left) and TWIMS (right) chromatograms and chronograms, respectively, for m/z 190.0352. A) and B) are from the 10^{-3} M mixture of AAs and AHAs that underwent 1 environmental cycle. C) and D) are from the raw discharge solution of ED experiment #3 (i.e. did not undergo cycling). E) and F) are from ED experiment #3 solution that underwent 1 cycle at 65 °C. G) and H) are from ED experiment #3 solution that underwent 1 cycle at 75 °C. I) and J) are from ED experiment #3 solution that underwent 1 cycle at 85 °C. K) and L) are from ED experiment #3 solution that underwent 1 cycle at 95 °C. Small differences in analyte retention times were observed for samples and dried down monomer standards due to the complexity of the samples.

Figure 6.10 shows an example analysis of the $[M-H]^-$ ion of 248.0406, regarding the one-time cycling of a discharge solution from ED experiment #4 at 65 °C, 75 °C, 85 °C, and 95 °C. When compared to a 10^{-3} M solution of AAs and AHAs that underwent one environmental cycle at 85 °C, there was a small UPLC-MS peak in each of the cycled solutions, perhaps representing the malic acid-aspartic acid dimer (Figure 6.10). The control sample of ED experiment #4 that underwent zero environmental cycles did not show a peak at m/z 248.0406 in either the UPLC-MS data (Figure 6.10.C), or the TWIMS data (Figure 6.10.D). It is worth noting that there was a small difference in retention times and drift times between analyte peaks in the samples, and the analyte peak in the cycled 10^{-3} M mixture of AAs and AHAs. This could potentially be explained by the complexity of the discharge samples and the low abundance of the species. It is expected that as the signal becomes weaker, the noise will influence the consistency of the signal progressively more, contributing to observed differences in retention times and drift times. The signal intensity for the $[M-H]^-$ ion at m/z 248.0406 was too low to allow MS/MS confirmation or precursor ion identification. Consequently, the detection of the malic acid-aspartic acid dimer from the environmental cycling of sample from ED experiment #4 described here, is categorized as tentative.

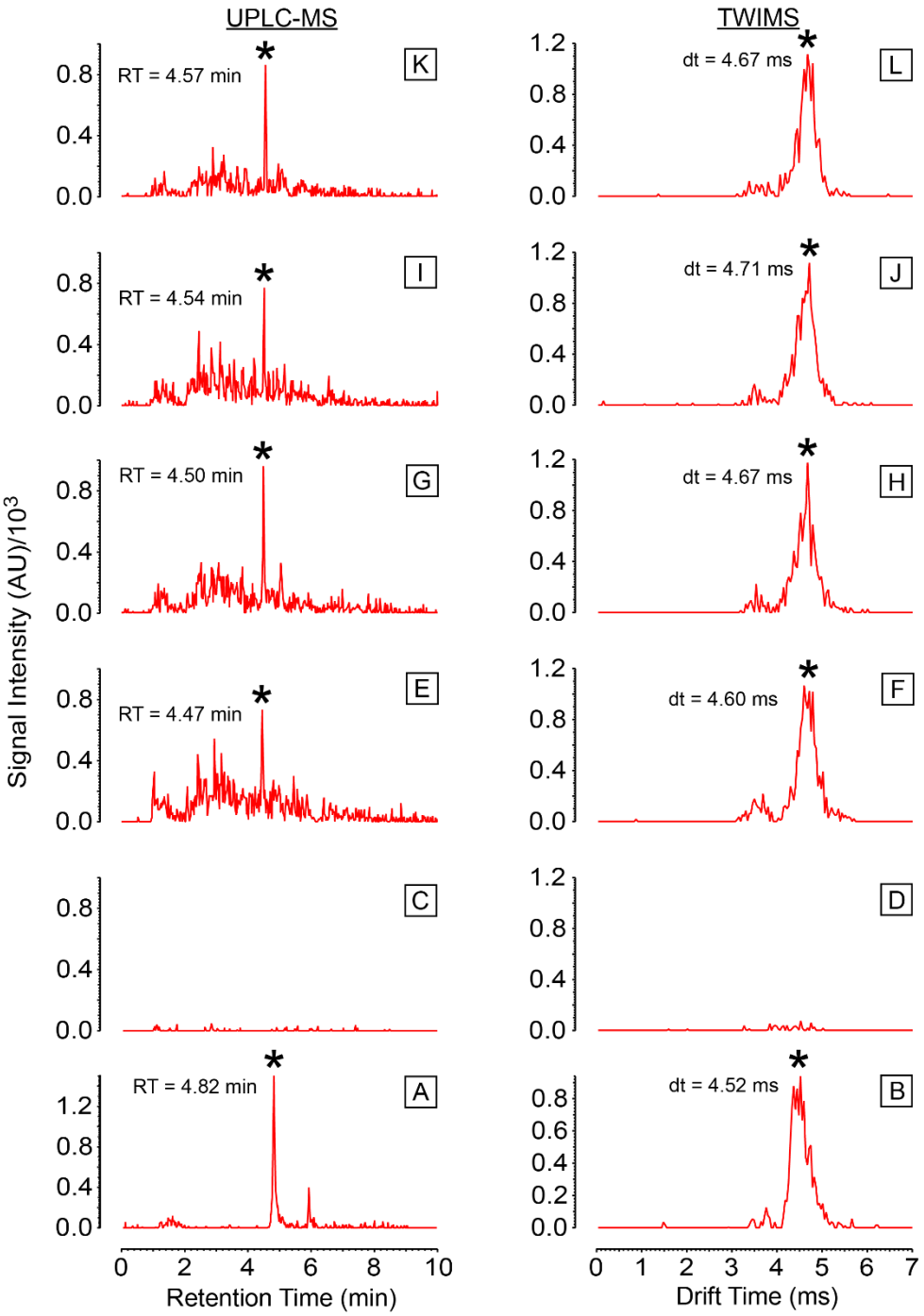

Figure 6.10. UPLC-MS (left) and IMS (right) chromatograms and chronograms, respectively, for m/z 248.0406. A) and B) are from the 10^{-3} M mixture of AAs and AHAs that underwent 1 environmental cycle. C) and D) are from the raw discharge solution of ED experiment #4 (i.e. did not undergo cycling). E) and F) are from ED experiment #4 solution that underwent 1 cycle at 65 °C. G) and H) are from ED experiment #4 solution that underwent 1 cycle at 75 °C. I) and J) are from ED experiment #4 solution that underwent 1 cycle at 85 °C. K) and L) are from ED experiment #4 solution that underwent 1 cycle at 95 °C.

6.4.3 *Acid-Vapor Hydrolysis Experiments*

Acid-vapor hydrolysis experiments were performed on ED samples produced here to determine if the preliminarily detected didepsipeptides contained an amide linkage. Because preliminarily detected didepsipeptides had signal intensities insufficient for proper MS/MS analysis and confirmation of compound identity, acid-vapor hydrolysis will provide additional evidence to support the preliminary detection of didepsipeptides. To illustrate, if the signal corresponding to the didepsipeptide preliminarily detected in the unhydrolyzed sample extract is also present in the hydrolyzed sample extract, this finding would serve as evidence to suggest that the preliminarily detected didepsipeptide did not contain an amide linkage that would have otherwise been cleaved during hydrolysis. Therefore, it could be concluded that the preliminarily detected didepsipeptide is not an amide-linked didepsipeptide. Conversely, if the signal corresponding to the didepsipeptide preliminarily detected in the unhydrolyzed sample extract is *not* present in the hydrolyzed sample extract, this finding would serve as evidence to suggest that the preliminarily detected didepsipeptide did indeed contain an amide linkage that was cleaved during hydrolysis. Therefore, it could be concluded that the preliminarily detected didepsipeptide was an amide-linked didepsipeptide.

This chapter will discuss data collected from the analysis of the hydrolyzed and unhydrolyzed extracts of ED Experiment #1, to provide an example of how acid-vapor hydrolysis of ED samples can serve as an additional test in an effort confirm the detection of didepsipeptides espousing from ED experiments.

Figure 6.7 demonstrates the detection of the glycolic acid-aspartic acid/glycine-malic acid didepsipeptides in cycled solutions from ED Experiment #1, based on LC

retention time, TWIMS drift time, and accurate mass. After subjecting these sample solutions to acid-vapor hydrolysis, the hydrolyzed extracts were analyzed by UPLC-Q-TWIMS-TOF-MS. A comparison of the UPLC-MS analyses of the hydrolyzed and unhydrolyzed extracts from ED Experiment #1 that underwent 0 – 4 cycles reveals that the signal corresponding to the glycolic acid-aspartic acid/glycine-malic acid didepsipeptides at m/*z* 190.0352 is detectable in the unhydrolyzed samples, but ***not*** the hydrolyzed samples (Figure 6.11).

These findings serve as evidence that the peaks detected at m/z 190.0352 in the unhydrolyzed samples did, in fact, contain a peptide bond. The presence of a peptide bond contributing to the signal observed in the unhydrolyzed samples, at a m/*z* and retention time consistent with that of the glycolic acid-aspartic acid/glycine-malic acid didepsipeptides, corroborates the preliminary detection of these didepsipeptides in the unhydrolyzed extracts.

Despite this added evidence for the detection of the glycolic acid-aspartic acid/glycine-malic acid didepsipeptides, it remains unclear if the observed signal is due to the glycolic acid-aspartic acid didepsipeptide, the glycine-malic acid didepsipeptide, or both species. Comparison of the analysis of ED solutions to the analysis of model mixtures unfortunately does not provide clarity on the matter, as the UPLC-MS signal for m/*z* 190.0352 in the model mixture only yields 1 peak (Figure 6.4). Therefore, it is unclear if the synthesis of both didepsipeptides is encouraged by environmental cycling but cannot be separated using the specified chromatography approach, or if one is favored relative to the other. This information can best be gained by performing an MS/MS

analysis on the observed peak in the unhydrolyzed samples, but the signal intensity was too weak for proper MS/MS analysis.

An additional uncertainty regarding the results from the acid hydrolysis experiment is the unknown peak observed at 5 minutes in Figures 6.11.D and 6.11.L. This peak is prominent in the hydrolyzed sample that underwent 0 simulated environmental cycles, but is substantially reduced in those samples that underwent 1, 2, and 3 cycles, before reappearing in the sample that underwent 4 cycles. It is possible that the hydrolysis of a larger species originally present in the ED solution produced a residue with a $[M-H]^-$ which matches that of the glycolic acid-aspartic acid/glycine-malic acid didepsipeptides, but elutes at a later retention time than these didepsipeptides. An MS/MS analysis would provide critical information to understand what chemical components contribute to these unknown peaks, however, the intensities for these signals are insufficient for MS/MS analysis.

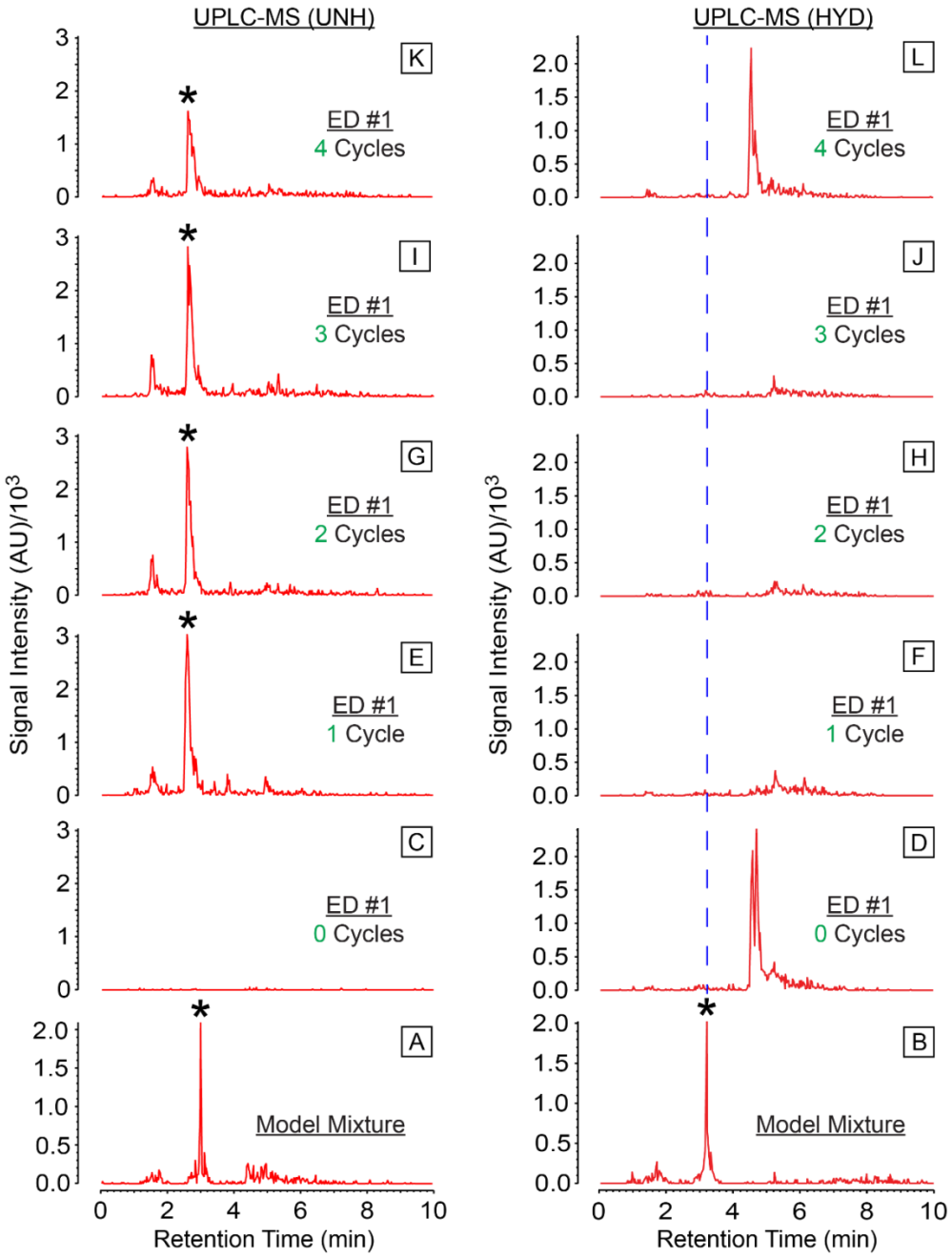

Figure 6.11. UPLC-MS chromatograms for m/z 190.0352 in the unhydrolyzed (left) and hydrolyzed (right) ED Experiment #1 samples. A) and B) are from the 10^{-3} M mixture of AAs and AHAs that underwent one environmental cycle. C) and D) are from the raw discharge solution of the ED experiment #1 (i.e. did not undergo cycling). E) and F) are from ED experiment #1 solution that underwent 1 cycle. G) and H) are from ED experiment #1 solution that underwent 2 cycles. I) and J) are from ED experiment #1 solution that underwent 3 cycles. K) and L) are from ED experiment #1 solution that underwent 4 cycles. Asterisks represent peaks used to tentatively identify the glycolic acid-aspartic acid dimer/glycine-malic acid dimer. Here, UNH = unhydrolyzed, and HYD = hydrolyzed.

6.5 Conclusions

In this chapter, a UPLC-Q-TWIMS-TOF-MS method was developed for the analysis of didepsipeptides in complex mixtures. Hexylamine was used as the ion-pairing agent to enable better chromatographic retention of target analytes, while ion mobility was utilized to provide an additional gas phase separation of dimers of interest. Those species for which signal intensity was highest were selected for CID in the transfer cell to facilitate MS/MS analysis and didepsipeptide sequencing.

To evaluate the performance of the developed method, a combined standard of the AAs glycine, alanine, aspartic acid and glutamic acid, and the AHAs glycolic acid, lactic acid, malic acid, and α-hydroxyglutaric acid, where each analyte was present at a concentration of 10^{-3} M, was prepared and underwent one environmental cycle at 85 °C to generate didepsipeptides. When applied to this mixture, the method developed here was able to retain target didepsipeptides chromatographically, as well as provide gas phase separation of many analytes of interest. After one environmental cycle, a total of ten species were present at sufficiently high concentrations to be selected for MS/MS fragmentation and confirm compound identity. Of these 10 analytes, 4 were monomers and 6 were dimers. The 4 monomers confirmed by MS/MS were aspartic acid, malic acid, glutamic acid, and α-hydroxyglutaric acid. The 6 dimers confirmed by MS/MS were dimalic acid, and the malic acid-alanine, α-hydroxyglutaric acid-alanine, malic acid-glutamic acid, α-hydroxyglutaric acid-aspartic acid, and α-hydroxyglutaric acid-glutamic acid dimers.

A series of prebiotic simulation experiments were performed and their samples were subsequently cycled in an attempt to induce co-polymerization of AA and AHA

monomers generated during the course of the experiments. Included in this sample cohort were solutions from a reproduction of Stanley Miller's 1958 cyanamide spark discharge experiment. Environmental cycling of discharge solutions was conducted at 65 °C, 75 °C, 85 °C, and 95 °C. Analyzed ED samples were subjected to 0 - 4 cycles.

The analyses of these mixtures revealed evidence for the formation of glycolic acid-aspartic acid/malic acid-glycine dimer and the malic acid/aspartic acid dimer.

This work stands as the first development of an analytical tool to use UPLC, TWIMS, and high resolution tandem MS in an effort to target didepsipeptides in complex mixtures stemming from prebiotic simulation experiments. Exposing a neat, 1 mM standard solution of AHAs and AAs demonstrates that co-polymerization of these monomers occurs readily to generate didepsipeptides. Cycling only generates a select few didepsipeptides at sufficiently high abundances to be selected for MS/MS fragmentation and sequencing, with others detected and matched to retention time, drift time, and m/*z* values. Furthermore, the evidence presented in this chapter underscores that while co-polymerization of AAs and AHAs is steadfast in neat solutions, as shown in this chapter and in the literature[10], this polymerization chemistry appears to be much more complex in mixtures generated from prebiotic simulation experiments. Furthermore, the concentrations at which any didepsipeptides that may be formed from environmental cycling of ED mixtures are not substantial enough to be accompanied by a sufficiently large signal intensity needed for fragmentation to confirm compound identity.

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CHAPTER 7: CONCLUSIONS

7.1 Abstract

The central theme of this thesis has focused on one of the foremost challenges facing the origin of life and prebiotic chemistry communities, biomolecule polymerization under abiotic conditions. A number of efforts have been undertaken in this body of work to extend the boundaries of the current understanding of possible prebiotic polymerization pathways.

7.2 Conducting Miller-Urey Experiments

7.2.1 *Overview*

Perhaps the most fundamentally important aspect of conducting prebiotic chemistry research is the need for conducting prebiotic simulation experiments. Many times in the past, results had been reported from the analysis of complex mixtures generated by experiments designed to mimic a primitive environment, however, a protocol detailing how to conduct prebiotic simulation experiments remained absent in the literature. Presented here is the first establishment of a step-by-step method to correctly and safely conduct prebiotic simulation experiments. One of the crucial steps that have been exhaustively explained includes the removal of ambient air, particularly O_2, from the reaction flask. While simulating a lightning discharge, via the use of an electrical spark, combustible gases (e.g. CH_4, NH_3) in the presence of O_2 will induce an explosion. Such an undesirable outcome is crucial to avoid, and this thesis teaches the experimenter how to do so by properly evacuating the reaction vessel. Upon completion of the prebiotic simulation experiment, it has been demonstrated how to conduct an

analysis for biomolecules, namely AAs, formed from the experiment. The analytical method of choice for this endeavor is a combination of OPA/NAC derivatization of samples, followed by HPLC separation and both fluorescence and mass spectral detection of target analytes. The use of derivatization in this approach is very beneficial because traditional LC-MS analysis of small organic compounds is inherently challenging. Small analytes are typically minimally retained using standard LC approaches, and mass spectrometers typically struggle to analyze low molecular weight species. However, the use of a derivatization agent, like OPA/NAC, helps overcome such hurdles by covalently linking a bulky molecular group to the target analyte, thereby enhancing chromatographic retention and avoiding the low mass cutoff held by mass spectrometers.

7.2.2 *Future Directions*

Future research directions associated with the performance of prebiotic simulation experiments are many. For example, the possibility that tungsten from the electrodes is sputtered during the spark discharge and ultimately impacts the chemical reactions that take place inside the reaction vessel, has been largely ignored. Additionally, the degree to which the glass surfaces of the reaction flask may serve as sites of chemical reactivity warrants investigation. But perhaps the largest and most intriguing uncertainty of these experiments pertains to the gas and particle phase chemistry. As has been stated in this thesis, there have been numerous explorations into the nature of the aqueous phase chemistry, yet the aqueous phase chemistry is likely preceded by the gas and aerosol phase reactions in the headspace of the reaction vessel. Unfortunately, there remains a large void in the literature, as it pertains to this gas phase chemistry. It is likely that simple, semi-volatile organic compounds are produced in the gas phase, which can then

partition into nearby aerosols. Once inside ambient particles, these species can undergo diverse chemical reactions, and can also be transported to the liquid reservoir below, before further chemistry occurs to eventually form biomolecules. However, the exact nature of the gas and aerosol phase chemical mechanisms responsible for the production of important biomolecule precursors remains misunderstood.

7.3 Comparison of Biomolecule Synthesis in MU Experiments and Simulated Titan Conditions

7.3.1 *Overview*

This body of work compares the synthesis of AAs in MU experiments and tholins produced by simulating conditions on Titan, the largest moon of Saturn. The protocol established in Chapter 2 of this thesis, and reported in the literature,[1] was used to conduct $CH_4/N_2/H_2O$ ED experiments simulating a variety of possible primitive terrestrial environmental scenarios, based on the work of.[2] Titan tholins were produced using the experimental protocol outlined by Neish et al.[3] Experimental samples were prepared for analysis by OPA/NAC derivatization, and then analyzed by UPLC-FD/TOF-MS. The results of these analyses clearly demonstrate the similarity of these two types of experiments, as it pertains to their respective syntheses of AAs. The majority of AAs detected in the solutions produced by these ED experiments were similarly detected in the hydrated Titan tholins. This finding highlights that molecules important for life can be similarly synthesized under highly dissimilar conditions, mimicking a number of different possible celestial environments. It also speaks to the robust nature of the synthetic mechanisms at play.

7.3.2 *Future Directions*

Much work needs to be done to better understand Titan chemistry, with implications to the habitability of Saturn's largest moon. Many fundamental chemical properties have yet to be explored under Titan conditions. To illustrate, the surface of Titan is ~95 K[4, 5] and covered by methane/ethane lakes.[6] However, it remains poorly understood if Titan is capable of sustaining the chemistry necessary to produce signs of life as we know it. For example, the reactivity and solubility of simple molecular precursors (e.g. aldehydes, ketones) needed to form organic compounds important for life is poorly constrained under Titan conditions. Furthermore, questions remain regarding the plausibility that molecular polymerization could occur under Titan conditions, given the exceedingly low temperatures and hydrocarbon-rich solvent sources. Much also needs to be explored regarding the possibility that solid state chemical synthesis could take place on the surface of Titan, if the hydrocarbon lakes of Titan do not sufficiently accommodate chemical reactivity. Alternatively, the shorelines of these hydrocarbon lakes could serve as a potential site at which solid materials (i.e. minerals) may come in contact with the hydrocarbon liquid on the surface of Titan to encourage dissolution and subsequent reactivity of chemical species. This possibility deserves further exploration in future Titan simulation experiments.

Beyond improving the knowledge of Titan chemistry, there are important improvements that need to be made, analytically, to provide a needed capability to detect AAs in complex mixtures. The method used in Chapter 3 of this thesis provides unambiguous detection of many primary AAs, yet it is not without drawbacks. For example, the technique does not detect diamines and molecules containing secondary

amines. It would be beneficial for the objectives of future Solar System and prebiotic chemistry studies to have access to a derivatization technique that can target diamines, primary amines, and secondary amines within a single analytical run. This would enable the analysis of a wider range of amine-containing biomolecules in complex mixtures than currently exists in the analytical chemistry literature.

7.4 The Miller Cyanamide Spark Discharge Experiment

7.4.1 *Overview*

The synthesis of polymerized biomolecules in prebiotic simulation experiments has long-since plagued the prebiotic chemistry community. A recently discovered set of archived samples produced by the late Stanley L. Miller from his 1950s spark discharge experiments contained samples from a 1958 experiment he performed that addressed the biomolecule polymerization issue. In this experiment, Miller sparked a gas mixture of CH_4, NH_3, and H_2O, while periodically introducing the plausible prebiotic condensing reagent, cyanamide. For unknown reasons, Miller had never reported an analysis of these samples, yet these old samples were recently analyzed for the first time, and done so using modern analytical instrumentation. A combination of liquid chromatography, ion mobility spectrometry, and high resolution mass spectrometry were implemented to detect the immediate products of AA polymerization, dipeptides and DKPs, in these samples.

The analysis revealed that dipeptides and DKPs were both detected in Miller's original samples. However, given the age of these samples, it was possible that storage conditions could have influenced the type and abundance of compounds in the sample mixture. To address this concern, the experiment was repeated to compare the analysis of

fresh samples to that of old samples. Analyzing samples from the repeated experiment confirmed the synthesis of dipeptides and DKPs, corroborating the findings from the analysis of the original samples.

It was previously considered that cyanamide-mediated AA polymerization was an acid catalyzed reaction, however, the pH of the discharge solution was ~10, due to the use of NH_3 as an experimental starting material. Therefore, the findings of dipeptides and DKPs in both the original and repeated cyanamide spark discharge experiment conflicted with previously held perceptions of cyanamide-mediated AA polymerization. Consequently, aqueous solution heating experiments were performed to search for an explanation for how cyanamide can induce AA polymerization in a non-acidic pH regime. The results from these experiments revealed that precursors generated during the Strecker synthesis of AAs over the course of the spark discharge experiment, the amino acid amide and aminonitrile, play a very important role in facilitating AA polymerization under basic conditions. To illustrate, when an AA and cyanamide are in the presence of an amino acid amide or aminonitrile, peptide synthesis is markedly enhanced, compared to when an amino acid amide or aminonitrile is not present. Furthermore, under basic conditions, cyanamide readily dimerizes to dicyandiamide, which can also react with an AA, in the presence of a Strecker synthesis intermediate, to yield peptides. Additionally, dicyandiamide proved to be a more effective condensing reagent than cyanamide. An implication of this study is that Miller's 1958 cyanamide spark discharge experiment marked the first effort to study a prebiotic condensing reagent for its implications to the origin of life. While it remains unclear how Miller got the idea to study cyanamide for its potential to condense AAs and generate peptides before anyone else had the idea, the

conclusions remain that Miller's 1958 cyanamide spark discharge experiment has expanded our knowledge of the range of environmental conditions that could have supported peptide synthesis on the early Earth.

7.4.2 *Future Directions*

One noticeable result that was obtained from this study was that cyanamide-mediated AA polymerization readily produced DKPs, and minimal amounts of tri- and higher order peptides. Therefore, AA polymerization is apparently limited when cyanamide is used as the polymerization reagent. However, additional condensing reagents exist as alternatives. For example, COS has been proposed as a plausible prebiotic condensing reagent.[7] Yet, it remains unclear how efficiently COS facilitates polymerization with an array of AAs that may have partially composed the prebiotic chemical inventory. To address this concern, more robust experiments should be conducted where COS is in the presence of AAs to better understand polymerization of AAs in the presence of carbonyl sulfide. Such experiments should be conducted at a variety of pH regimes, as well, to constrain the pH at which such polymerization chemistry yields optimal peptide synthesis. Additionally, it would be recommended to conduct a prebiotic simulation experiment that includes COS. Such an experiment would aid the assertion of COS to readily facilitate AA polymerization within a complex mixture.

7.5 Quantitation of α-Hydroxy Acids in ED Experiments

7.5.1 *Overview*

The synthesis of AAs had been well documented in the prebiotic chemistry literature, however, their AHA analogs had not been specifically targeted for during

analysis of prebiotic simulation experiment al samples since Miller's work in the 1950s.[8, 9] Considering the findings of Forsythe et al.,[10] which suggest that AAs and AHAs could yield depsipeptides, and effectively mitigate DKP formation, it became necessary to constrain the AHAs that could have been present on the early Earth and participated in this reported chemistry. Additionally, there was a gap in the literature, with respect to analytical platforms capable of quantifying a wide array of AHAs. Consequently, the objectives of this chapter were to conduct prebiotic simulation experiments to synthesize AHAs, as well as developing an analytical technique to quantify AHAs generated by such experiments.

The work discussed here involved performing ED experiments containing gas mixtures of CH_4, H_2O, and N_2 or NH_3. A targeted, quantitative method was developed using UPLC/QqQ-MS. Chromatographic retention of target analytes was facilitated by the use of hexylamine as an ion-pairing agent. The method developed here was the first mass spectrometry-based approach that demonstrated the ability to analyze a double-digit number of AHAs within a single chromatographic run. The detection limits offered by the method were in the nanomolar concentration range for each AHA tested. When applied to solutions from ED experiments, small concentrations (typically on the order of a few, to tens of micromolar) of the AHAs glycolic acid, lactic acid, malic acid, and α-hydroxyglutaric acid were detected under a variety of simulated primitive ocean-atmosphere systems. As a result, it was suggested that these AHAs, and their corresponding AAs, glycine, alanine, aspartic acid, and glutamic acid, respectively, be focused on in future studies regarding depsipeptide synthesis.

7.5.2 *Future Directions*

Despite the analytical achievements made in this chapter, more can be done to improve the capabilities of detecting AHAs in complex mixtures. First, it would be ideal to develop a method that can not only detect AHAs, but also provide enantiomeric separation of AHAs with chiral centers. This type of method would be particularly impactful to the prebiotic chemistry field, as well as the meteoritic sciences field, where analyzing building blocks formed by extraterrestrial synthesis is feverishly studied. Quantitation of enantiomeric pairs is regularly used in these fields to discern between terrestrial contamination of target analytes with chiral centers, and extraterrestrial synthesis, an important distinguishing characteristic. Additionally, AHAs tend to be synthesized in relatively low concentrations due to the pH of the discharge mixture. It is likely that additional AHAs were synthesized in the experiments performed yet went undetected due to their low abundances. In order to better target these smaller concentrations of these small analytes, a non-mass spectrometry-based method may be well suited to overcome such an analytical challenge For example, microchip capillary electrophoresis with laser-induced fluorescence[11] has demonstrated an innate ability to effectively target small molecules, separate them from one another within complex mixtures, and detect these species at very low concentrations. It should be stated that this type of analytical approach is capable of performing separations on the order of seconds, and the use of a fluorescent detection scheme helps enable detecting individual enantiomers of chiral centers, provided an appropriate fluorescent dye is used. This is in contrast to a mass spectrometry-based detection methods, which cannot differentiate enantiomers of chiral centers when non-derivatization-based approaches are used.

7.6 The Search for Didepsipeptides in Prebiotic Simulation Experiments

7.6.1 *Overview*

The research detailed in this exploration outlines the first technique developed to analyze didepsipeptides using a combination of liquid chromatography, ion mobility spectrometry, and high resolution mass spectrometry, including tandem mass spectrometry. Target didepsipeptides were chromatographically retained using hexylamine as an ion-pairing agent. Proof-of-concept experiments were performed, first using dried down, neat, aqueous solutions of AA and AHA monomers, to produce their co-polymerization products. After the method was developed, it was then applied to analyze complex mixtures generated by a series of ED experiments, including a reproduction of Miller's cyanamide spark discharge experiment. The samples originating from these experiments were then subjected to environmental cycles that ranged in temperature from 65 °C - 95 °C, and in frequency from 0 - 4 cycles. Analyses of these solutions resulted in the tentative detection of the glycolic acid-aspartic acid dimer and the malic acid-glycine dimer. The abundances of these species were too low for robust identification confirmation via tandem mass spectrometry. These findings illustrate that AA/AHA co-polymerization can yield didepsipeptides in a neat solution, but highlights that this polymerization chemistry is not carried out as readily when exposing complex mixtures from ED solutions to mimicked environmental cycling. The concentration of AAs and AHAs produced in a prebiotic experiment are typically far less than the concentrations of those species used by Forsythe et al.,[10] and the complexity of discharge solutions are far greater, likely convoluting the polymerization pathway outlined by

Forsythe et al.,[10] and resulting in markedly diminished products of AA/AHA co-polymerization.

7.6.2 *Future Directions*

From an analytical standpoint, perhaps the most important step in the next direction would be to acquire depsipeptide standards. Currently, depsipeptide standards are difficult to synthesize, leading to their scarce commercial availability, and high costs. However, it would behoove the Center for Chemical Evolution, should it choose to continue to pursue questions related to possible prebiotic depsipeptide synthetic pathways, to employ a team of peptide synthetic chemists to develop the protocols necessary to make high purity depsipeptide standards. Once depsipeptide standards become available, many challenges associated with analyzing samples for depsipeptides will become much more manageable. To illustrate, chromatography experiments could be conducted to confidently improve depsipeptide retention and separation. Chromatography of depsipeptides would likely be enhanced by the development of a derivatization technique with specificity for depsipeptides, as this chapter demonstrates that even when using an ion-pairing agent, co-elution of target analytes remains a challenge. Achieving this chromatographic feat would greatly mitigate the possibility of ion suppression acting to reduce analyte signal intensity, and in turn, contributing to a heightened likelihood of conducting MS/MS experiments on a wider range of depsipeptides. Depsipeptide standards would also provide the opportunity to develop an improved analytical method for the *unambiguous* detection of depsipeptides using LC, IMS, and accurate mass MS/MS, as well as provide a reference by which to perform quantitative measurements of these analytes.

Steps can be taken in an effort to improve the likelihood of detecting depsipeptides originating from prebiotic simulation experiments. First, ED experiments should be conducted under more neutral pH conditions than were performed here, as the synthesis of α-AAs and AHAs is near unity when the pH of the aqueous solution is close to 7.[12, 13] This can be achieved by buffering the aqueous phase inside the ED flask, and by using N_2 as a starting gas, as opposed to NH_3. The ED experiments performed in this thesis had slightly basic pH values, which is amenable to the synthesis of α-AAs, and disfavors the formation of AHAs. Consequently, the abundance of AHAs available in the ED mixtures, from which to potentially synthesize depsipeptides, is inherently restricted, therefore contributing to minimal didepsipeptide synthesis upon cycling of ED experimental samples.

Second, prebiotic experiments can be performed and subsequently subjected to freezing. Such a eutectic solution has demonstrated the ability to concentrate organic compounds and facilitate polymerization to yield short peptides.[14] Solutions generated from ED experiments, both in the presence of condensing reagents and without, can be stored at low temperature to form a eutectic brine in an attempt to concentrate the α-AAs and AHAs generated by the experiments. This type of analyte concentration process could be capable of efficiently inducing co-polymerization of these two classes of monomers to yield simple depsipeptides.

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APPENDIX A. CURRENT PERCEPTIONS OF THE EARLY EARTH'S ATMOSPHERE

The atmospheric composition of the early Earth holds tremendous implications for the origin of life on Earth. It is likely the atmosphere of the primitive Earth largely influenced molecular synthesis and the prebiotic chemical inventory.[1, 2] Such gaseous species would have most likely been provided by magmatic outgassing from the Earth's interior, with volcanic vent systems serving as a major transport mechanism for these volatiles.[3] However, the composition of magmas beyond 3.85 bya is very limited, or non-existent, preventing direct estimations of the early atmospheric composition.[4]

When the seminal work of Miller and Urey was published in the early 1950s,[5] it was thought that the early Earth's atmosphere was heavily reducing, containing CH_4, NH_3, H_2O, and H_2 as major constituents.[6] The perception was previously held that formation of the Earth occurred slowly, and that much of the volatile gases of the Earth were trapped inside the planet, largely due to the cold interior of the Earth.[7, 8] Not until the interior of the Earth became warmed by radioactive decay, was it though that the trapped volatiles would have been released via volcanism.[3] Upon release, these gases were speculated to be heavily reducing, (similar to the mixture employed by Miller[5]), becoming more oxidized after the development of the Earth's core, at which point the volcanic emissions would have been dominated by N_2, H_2O, and CO_2, with lesser amounts of CO and H_2.[8]

Since these early studies of planetary accretion, it has been proposed that the formation of the Earth actually took place relatively quickly and its interior was initially quite hot because of impact events.[9, 10] Such impact events likely released volatiles

trapped within the Earth,[11, 12] and are thought to have contributed to the formation of a primitive atmosphere with large amounts of water vapor during planetary accretion.[13, 14] Furthermore, it has been proposed that the formation of Earth's core likely occurred concomitantly with planetary accretion[15, 16] and that volcanic outgassing could have been composed of oxidized species as far back in earth's history as beyond 4 bya.[4, 17]

Consequently, the current perceptions of the prebiotic Earth's atmospheric composition is different from the one imitated by Miller.[5] A heavily reducing atmosphere is no longer considered to have been likely on a global scale on the primordial Earth. Instead, a weakly reducing or neutral primitive terrestrial atmosphere is considered to have been more likely on a global scale. Such an atmosphere would have been comprised of major constituents, including CO_2, N_2,[3] CO, and H_2O, with minor amounts of H_2, H_2S, sulfur dioxide (SO_2) and CH_4.[18]

While reducing atmospheric conditions may have been unlikely on a global scale on the early Earth, they might have been present within smaller scales,[19, 20] including island-arc systems.[21, 22] These types of microenvironments could have been important locales capable of fostering a suite of very powerful prebiotic chemical reactions to produce large quantities of molecules important for life.[21, 22] Even under neutral atmospheric conditions, biomolecules have shown to be efficiently synthesized, based on laboratory studies.[18]

A.A.1 *References*

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APPENDIX B. THE MECHANISM OF ION-PAIR CHROMATOGRAPHY (IPC)

Chapters 5 and 6 focused heavily on the development of analytical methods to detect AHAs and didepsipeptides, respectively. In both cases, part of the analytical platform employed was LC, specifically to separate target analytes prior to mass spectral analysis. Initially, it was found that these target analytes experienced minimal retention and separation when using a variety of direct LC approaches with standard eluents. However, AHAs and didepsipeptides experienced enhanced retention and separation after employing the use of hexylamine as an ion-pairing agent added to the mobile phase[1].

Although the mechanism by which hexylamine acts as a suitable ion-pairing agent for the retention and separation of AHAs and didepsipeptides was not discussed in Chapters 5 and 6 of this thesis, the operating principles of IPC remains inherently germane to the understanding of the observed chromatographic results. Therefore, this section of the Appendix will be devoted to overviewing the details of the mechanism by which IPC facilitates chromatographic retention and separation of analytes that are challenging to separate using traditional LC approaches.

First, it is important to review fundamental pillars of LC, which will explain how target analytes were separated. In chapters 5 and 6 of this thesis, a C18 column was implemented. C18 columns are equipped with a stationary phase composed of alkylated silica with a chain length of 18 carbon atoms. C18 columns are typically used with polar solvents. Consequently, a mostly aqueous solvent system was used in Chapters 5 and 6 of this thesis. The non-polar nature of the packing material used in C18 columns makes the stationary phase hydrophobic. As a result, compounds in the mobile phase that possess

more non-polar, hydrophobic characteristics will interact more strongly with the stationary phase than molecules with more polar, hydrophilic characteristics. Therefore, more non-polar, hydrophobic molecules will spend more time in the stationary phase, resulting in enhanced analyte retention, while more polar, hydrophilic molecules will spend more time in the mobile phase, resulting in decreased analyte retention. The AHAs and didepsipeptides targeted in Chapters 5 and 6, respectively, are fairly polar, hydrophilic molecules, due to the presence of carboxylic acid functional groups in these species. These inherent molecular properties of AHAs and didepsipeptides caused the initial observation of their sparing interaction with the stationary phase, resulting in minimal retention and separation.

Through the use of IPC, however, these analytes were able to experience enhanced retention and separation. IPC is a chromatographic approach that is useful for the separation of polar analytes. In IPC, the mobile phase used to transport the target analytes through the chromatography column, is doped with relatively small (typically millimolar) concentrations of an additive known as an ion-pairing agent. Suitable ion-pairing agents are typically relatively large ionic compounds that possess the following characteristics: 1) a hydrophobic moiety and 2) a charge opposite that of the target analyte. The hydrophobic moiety of the ion-pairing agent allows it to interact strongly with the hydrophobic stationary phase of the chromatographic column, while the charge oppose to that of the analyte of interest, allows the ion-pairing agent to interact strongly with the target species. The mobile phase implemented during IPC also typically involves the use of counter-ions, which serve important purposes. As has been shown in the

literature, counter-ions are often used at concentrations on the order of tens of millimolar, and depending on the concentration of the counter-ion, can alter analyte retention.[2]

The IPC protocol implemented in Chapters 5 and 6 of this thesis played a major role in influencing analyte retention. It is important to note that analyte retention is determined by thermodynamic equilibria,[3-5] not kinetic processes, which influence analyte peak widths.[4] The IPC protocol used involved a dual buffer system. Buffer A was 5 mM hexylamine in water, and buffer B was 90:10 methanol:10 mM NH_4OAc in water. Therefore, the pairing agent (P^+) was hexylamine, an amine-containing isomer of hexane, used to form an ionic interaction with the compounds of interest, and is readily positively ionized in solution. The target analytes (A^-) were AHAs and didepsipeptides, which contain carboxylic acid moieties that are readily negatively ionized in solution. The counter ions used in buffer B are ammonium (NH_4^+) as the positive counter ion (C^+), and acetate (^-OAc) as the negative counter ion (C^-). The alkylated silica packing material of the C18 column served as the stationary phase (S).

The processes largely considered to be responsible for dictating the chromatographic thermodynamic equilibrium achieved have been discussed in detail in the literature,[2, 3, 6-13] but will be briefly summarized here. There are a handful of equilibria processes at work:

1) Adsorption of the analyte onto the stationary phase of the column:

$$A_m^- + C_m^+ + S_s \overset{k_1}{\leftrightarrow} (ACS)_s \quad (1)$$

2) Formation of an ion-pair in the mobile phase:

$$A_m^- + P_m^+ \overset{k_2}{\leftrightarrow} (AP)_m \quad (2)$$

3) Adsorption of the pairing agent to the stationary phase:

$$P_m^+ + C_m^- + S_s \overset{k_3}{\leftrightarrow} (PCS)_s \quad (3)$$

4) Adsorption of analyte and pairing agent, yielding an ion-pair in the stationary phase:

$$A_m^- + P_m^+ + S_s \overset{k_4}{\leftrightarrow} (APS)_s \quad (4)$$

As was the case for the AHAs and didepsipeptides focused on in Chapters 5 and 6 of this thesis, the analytes are in relatively low concentrations, while the concentration of the stationary phase was presumed to be constant, resulting in the following association:

$$[S] = [S_s] + [(PCS)_s] = constant \quad (5)$$

As a result, the addition of a pairing agent to the eluent can influence the retention of a target analyte via the following column capacity ratio (k') equation:

$$k' = \varphi[S]\frac{k_1[C_m^-] + k_4[P_m^+]}{\{1 + k_2[P_m^+]\}\{1 + k_3[P_m^+][C_m^-]\}} \quad (6)$$

where φ is the ratio of stationary phase to mobile phase. In equation (6), the term $k_1[C_m^-]$ is a result of the adsorption of the analyte directly onto the stationary phase (i.e. in the absence of the pairing agent), which, when the pairing agent has been added at sufficient concentrations to the eluent, has a negligible quantity. Furthermore, it can be seen that an increase in the formation of an ion-pair in the mobile phase (i.e. the term containing k_2), or the adsorption of the pairing agent into the stationary phase without the target analyte (i.e. the term containing k_3), both act to reduce analyte retention. ***Therefore, one can deduce that the overarching process responsible for the enhancement of the retention of A^- is a result of the adsorption of the analyte onto the stationary phase via the formation of an ion-pair.***

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APPENDIX C. BIASES OF ED EXPERIMENTS

A.C.1 *Generation of an ED in Prebiotic Simulation Experiments*

It is important to keep in mind that prebiotic simulation experiments, namely of the ED variety, are not without biases that may influence the products observed. Perhaps the most intriguing source of biases is the use of the ED, itself. In such experiments, lightning is mimicked via the use of an ED, produced by a high frequency generator, which is based on the operating principles of a resonance transformer (e.g. Tesla Coil) first developed by Serbian-American engineer and physicist, Nikola Tesla in 1891.[1-3] The manner in which such a device works has been studied extensively,[4-8] but will be briefly overviewed here. The generator used in this thesis is a Model BD-50E high frequency generator manufactured by Electro-Technic Products Inc. (Chicago, Illinois). The device contains an isolation transformer, capacitor, spark gap, and resonator coil. The isolation transformer functions to take a low voltage input from the electrical outlet, and convert it into higher voltage. This voltage is then delivered to the primary circuit, which includes the capacitor, spark gap, and resonator coil. By doing so, the isolation transformer charges the capacitor, which is designed to store electrical energy, until a sufficiently high charge has accumulated in the capacitor to initiate the transfer of electrical current across the spark gap. This surge of current is then transferred to the resonator coil assembly. The resonator coil contains two coils, a primary coil and a secondary coil. The electrical current that is sent across the spark gap first reaches the primary coil, which generates a magnetic field. This magnetic field induces a voltage in the secondary coil. The voltage pulse travels along the secondary coil at a frequency of 500 kHz. The net result is a building of increasingly higher voltage in the secondary coil, until the

accompanying electric field is strong enough to strip electrons off of gaseous species in the air, inducing an electrical arc across the two tungsten electrodes used in the experiments performed here.

A.C.2 *The Global Electrical Circuit and Lightning*

The generation of an electrical arc across tungsten electrodes does not accurately portray lightning discharge in the atmosphere. Extensive reviews on the processes influencing atmospheric lightning have been offered elsewhere,[9-14] but a short synopsis of select examples on the current Earth will be discussed here, with extrapolations made to the prebiotic Earth, where appropriate.

The Earth (both solid and liquid components) and the atmosphere of Earth are electrified on a near-constant basis. Earth's surface carries a net negative charge, while the atmosphere carries a net positive charge equal in magnitude to the charge carried by Earth's surface. The conductivity of the atmosphere increases on a roughly exponential basis as altitude increase, up to 60 km. Negative ions and positive ions produced by cosmic radiation are the primary charge carriers below 60 km. Above 60 km, free electrons become important components that influence conductivity. The presence of the geomagnetic field causes atmospheric conductivity above 80 km to change in magnitude, based on directionality factors.

The global electrical circuit is thought to be contained between the Earth and the ionosphere (~60 km – 1000 km in altitude), both of which serve as highly conductive layers. Furthermore, the global electrical circuit is considered to be initiated by thunderstorm activity, which transports current from the surface, to higher altitudes. Thunderstorms are most common over tropical and subtropical land masses during the

afternoon and evening. The more energy contained within thunderstorms, the higher the altitude that associated charge can be carried. These thunderstorms can be considered as generators that are dipolar current sources: negative source at the bottom of the thunderstorm cloud and positive source at the top of the thunderstorm cloud.

Select measurements have been made to quantify the flow of current from a thunderstorm cloud,[15] and it has been shown that currents can vary between 0.1 amperes and 6 amperes, but generally possess an average value of 0.5 amperes – 1 ampere.[16] Above a thundercloud is a "charging resistor", which is a conduction current that flows from the top of the thundercloud to the ionosphere, and is capable of charging the ionosphere to an electrical potential of ~+250 kV, relative to the Earth's surface.[17] Current flows upward from the top of a thunderstorm cloud, to the ionosphere. Given the high conductivity of the ionosphere, current is allowed to travel in a horizontal fashion, while experiencing negligible resistance. The electrical potential that exists between the ionosphere and Earth's surface induces an electrical current to be driven downward to the surface. Once at the surface, current can, again, travel freely in a horizontal direction, due to the conductive nature of Earth's surface. The circuit can be completed by transfer of current between the Earth's surface and the base of a thunderstorm cloud via convection, conduction, hydrometeor precipitation, and lightning discharges.[18] Although electrical resistance is not incurred when current travels horizontally, resistance can be experienced when current travels vertically. Such factors as columnar resistance between the Earth's surface and the ionosphere, and the potential difference of the ionosphere, are capable of serving as sources of resistance for vertically-traveling current.

Although land masses may not have been as prevalent on the prebiotic Earth as they are today,[19] thunderstorms were likely sufficiently common on the early Earth to enable the global electrical circuit to have occurred throughout Earth's history to deliver lightning discharges as a featured source of energy on the primitive Earth.[20]

A.C.3 *Aerosols and Lightning*

An intriguing factor that influences lightning production in the atmosphere is aerosols, a component that is not adequately represented by the use of a high frequency generator to produce a spark in the ED experiments. Aerosols at low and high altitudes can both influence lightning activity. Low altitude aerosols, such as marine- and continental aerosols, are contained within the planetary boundary layer (lowest few km of the atmosphere). High elevation aerosols are often produced by volcanoes that inject particles above the planetary boundary layer.

Aerosols are capable of serving as cloud condensation nuclei, which are known to influence atmospheric precipitation patterns. Consequently, it is possible that aerosols can indirectly influence the occurrence of lightning events, due to the correlation between precipitation and lightning activity.[21-23] An increase in cloud condensation nuclei can influence the size, phase, and concentration of cloud particles, which may in turn induce changes to microphysical processes occurring within a thundercloud, such as altering charge separation processes.[24] Furthermore, a greater concentration of aerosols will likely suppress the average droplet size, enabling more cloud water to separate electric charge, thereby facilitating an increased frequency of cloud-to-ground lightning discharges.[25]

Volcanic eruptions are capable of injecting aerosols into the upper troposphere and lower stratosphere.[11] Volcanoes are known to produce dust particles and sulfate

aerosols that can induce nucleation and form high altitude clouds.[11] Additionally, these sulfate aerosols change the conductivity of the atmosphere, and therefore the global electrical circuit.[11] Consequently, it is possible that on the primordial Earth, volcanic vent systems could have served as a source of high altitude sulfate aerosols that subsequently enabled the generation of lightning discharges by generating thunderclouds and altering the global electrical circuit to produce lightning.

The physical contributions of aerosols to the occurrence of lightning discharges can also be accompanied by a chemical contribution to the formation of biomolecules. Air-water interfaces, namely aerosols, are considered to have been vital microenvironments, within which, prebiotic chemistry could have been carried out on the primitive Earth.[26-29] Aerosols possess the ability to align and concentrate precursors of molecules important for life, and change the ionization of chemical species on the surface of the aerosol.[30-34] Studies of modern marine aerosols have discovered that amino acid precursors can be contained within these particles.[35]

Aerosols could have served as important chemical reactors on the early Earth. When combined with the potential of particles to change cloud microphysical processes and induce lightning discharges near volcanic vent systems on the primitive Earth, for example, aerosols could have served as important microenvironments to supply organic precursors, and facilitate the generation of lightning to serve as an energy source to drive chemical reactions and form molecules important for life. Within the ED experiments performed in this thesis, aerosols were likely present to aid in the transport of precursors from the gas phase, to the aqueous layer. However, the possibility that aerosols may serve as a potential conduit for an energy source is not replicated by these experiments.

Therefore, the use of an ED produced by a high frequency generator is not a fully accurate representation of natural lightning, particularly as it pertains to lightning mediated by such atmospheric components as aerosols.

A.C.4 *Metal Particles from ED*

The impact of metallic particles on the experiments performed here is another potential source of bias, and is also a remaining uncertainty of these prebiotic simulations. Tungsten electrodes were used for enacting the spark discharge, in part, because sputtering of tungsten is relatively minimal, and the element has a large thermal conductivity.[36-37] However, a major issue surrounding tungsten as a plasma facing material is that the self-sputtering yield of Tungsten is fairly high at relatively low energy levels.[38] Therefore, it remains a possibility that small amounts of tungsten are sputtered when the electrodes come in contact with the ED, thereby depositing tungsten particles onto the walls of the reaction flask, or in the aqueous layer during the course of the experiment. The deposition and accumulation of tungsten particles inside the reaction flask could have important impacts on the chemical products observed during analysis. For example, carbon is capable of reacting with a variety of carbon-bearing species[39] and can even act as a catalyst under certain circumstances.[40] The role of tungsten sputtering in spark discharge experiments designed to simulate early Earth conditions has been sparsely explored within the literature. The potentially profound implications of the ED inducing sputtering of the tungsten electrodes and possibly making tungsten available to carry out a variety of reactions during the experiment, warrants exploration. Such processes may be responsible for the formation of artifacts present during analytical processing of the discharge mixture.

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Johnson, A. P.; Cleaves, H. J.; Dworkin, J. P.; Glavin, D. P.; Lazcano, A.; Bada, J. L. (2008) The Miller Volcanic Spark Discharge Experiment. *Science*, 322, 404.

I'd like to use Figure 1C in an unmodified fashion. Furthermore, I'd like to use Figure S1 and Table S1, both in modified forms. Regarding using modified version of figures and tables, I read the following stipulation online:

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Thank you,
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Re: Request Permission to Use Modified Material

Jeffrey Bada <jbada@ucsd.edu>

Tue 5/31/2016 12:21 PM

Inbox

To:Parker, Eric T <erictparker@gatech.edu>;

Hi Eric, sorry I forgot about this. You have my permission to use what ever material in the Volcanic Paper you need for your thesis. Do you need this on formal letterhead or does this work? Let me know.

Parker, Eric T wrote:
> Hi Jeff,
>
>
> I think you may have been in the middle of moving when I sent this email,
> so this is a friendly reminder.
>
>
> Thanks,
>
> Eric
>
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> From: Parker, Eric T
> Sent: Monday, May 16, 2016 11:44:41 AM
> To: Jeffrey L. Bada
> Subject: Fw: Request Permission to Use Modified Material
>
>
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>
>
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> paper, in the intro chapter of my thesis. I am required to receiver
> permission from the author. I'd like to use an unmodified version of Fig
> 1C, and modified versions of figure S1 and table S1. I've attached the MS
> and SI, in addition to the modified versions of figure S1 and table S1, so
> you can compare and contrast. Please let me know what you think.
>
>
> Thanks,
>
> Eric
>
>
> _____

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Avital Braiman <avital.braiman@jove.com>

Tue 1/19/2016 9:52 AM

To:Parker, Eric T <erictparker@gatech.edu>;

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Best,

Avital

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Date: Mon, Jan 18, 2016 at 12:31 PM
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To: "editorial-office@jove.com" <editorial-office@jove.com>

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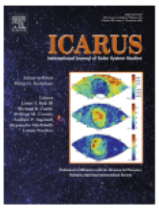

Title: Amino acids generated from hydrated Titan tholins: Comparison with Miller–Urey electric discharge products

Author: H. James Cleaves,Catherine Neish,Michael P. Callahan,Eric Parker,Facundo M. Fernández,Jason P. Dworkin

Publication: Icarus

Publisher: Elsevier

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